PB85-242527

Investigation on the Potential Environmental Hazards of Pesticidal Viruses. 1. Molecular Biology of 'Spodoptera frugiperda' Nuclear Polyhedrosis Virus. 2. Lack of Evidence for Possible Environmental Hazards

North Carolina Univ. at Chapel Hill

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

Jul 85

U.S. Department of Commerce
National Technical Information Service
NTIS

PB85-242527

EPA/600/1-85/018
July 1985

INVESTIGATION ON THE POTENTIAL ENVIRONMENTAL HAZARDS OF PESTICIDAL VIRUSES

I. Molecular Biology of *Spodoptera frugiperda* Nuclear Polyhedrosis Virus
II. Lack of Evidence for Possible Environmental Hazards

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Grant Number: R806210

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This study was conducted in cooperation with U.S. Department of Agriculture, Beltsville, MD 20705

Health Effect Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, NC 27709

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TECHNICAL REPORT DATA
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1. REPORT NO. EPA/600/1-85/018	2.
3. RECIPIENT'S ACCESSION NO. PB85 242527AS	
4. TITLE AND SUBTITLE Investigation on the Potential Environmental Hazards of Pesticidal Viruses	5. REPORT DATE July 1985 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) E-S. Huang, L. Loh, Y-M. Wu and E-C. Mar	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Cancer Res. Center & Dept. of Med. & Dept. of Microbiology & Immunology, School of Med., Univ. of North Carolina at Chapel Hill Chapel Hill, NC	10. PROGRAM ELEMENT NO. E104 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS USEPA, HERL, DBD, PTB RTP, NC 27711	13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES	

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Besides the study on the biopathology of a pesticidal virus, Spodoptera frugiperda nuclear polyhedrosis virus (SfNPV), we have also extensively studied the molecular structure of the genome of this virus justified on the need in developing non-hazardous universal pesticidal viruses. The complete set of virus DNA fragments have been cloned in pBR322 plasmid. This set of the recombinant plasmid is now available for further gene function study.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES 64
	20. SECURITY CLASS *(This page)*	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory, Research Triangle Park, NC and Cincinnati, OH conducts a coordinated environmental health research program in toxicology, epidemiology and clinical studies using human volunteer subjects. Wide ranges of pollutants known or suspected to cause health problems are studied. The research focuses on air pollutants, water pollutants, toxic substances, hazardous wastes, pesticides, and non-ionizing radiation. The laboratory participates in the development and revision of air and water quality criteria and health assessment documents on pollutants for which regulatory actions are being considered. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

F.Gordon Hueter,Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

Due to the environmental and ecological effects of toxic chemical pesticides, the usage of insect viruses have been considered as one of the alternatives for the control of agriculture insect pests. In fact in the past 3 decades, several baculoviruses have been used as viral pesticides for pest control. It has not been demonstrated to be hazardous to non-target organisms using the classical infectivity and morphological alteration as measuring factors. In this research project, we have further used molecular biological approaches to characterize the molecular structure of one of the insect viruses to investigate and elucidate the possible pathogenicity and oncogenicity of pesticidal viruses to human and other mammals at in vitro level. Our study suggests that the pesticidal virus Spodoptera Frugiperda (SF) can not productively infect human fibroblast or HEP-2 cell lines and can not induce morphological transformation of human fibroblast.

Besides the study on the biopathology of a pesticidal virus, Spodoptera frugiperda nuclear polyhedrosis virus (SfNPV), we have also extensively studied the molecular structure of the genome of this virus justified on the need in developing non-hazardous universal pesticidal viruses. The complete set of virus DNA fragments have been cloned in pBR322 plasmid. This set of the recombinant plasmid is now available for further gene function study.

This work was carried out in the Cancer Research Center and the Department of Medicine, University of North Carolina at Chapel Hill under the support of U.S. Environmental Protection Agency. This report is submitted in fulfillment of Grant Number R806210 by the University of North Carolina under the sponsorship of U.S. Environmental Protection Agency. The report covers the period June 10, 1978 to September 10, 1981.

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ACKNOWLEDGEMENTS

The experimental research material of this work was partly provided by Dr. Clinton Kawanishi of the U.S. Environmental Protection Agency at Research Triangle Park, NC (for different insect cell lines and SfNPV) and Dr. John J. Hamm of Southern Grain Insects Laboratory. The authors are indebted to both of them. We also thank Dr. Y.S. Huang of the U.S. Environmental Protection Agency for providing us the technical assistance and scientific advice.

Some of the experimental work reported here was carried out by Afshin Meymandi and S.M. Huong with skill and patience and their contribution is hereby acknowledged. The authors are also indebted to Miss Barbara Leonard for excellent secretarial assistance.

I. INTRODUCTION

In recent years, there has been great interest in industry and government in searching for the possible usage of insect viruses as an alternative in chemical pesticides in the control of agricultural insect pests. (Ignoffo, 1973 and 1975, Falcon 1976 and 1977). This great impetus to use viral pesticides is based on the environmental and ecological effects of toxic chemical pesticides. For example, the three most commonly used chemical insecticides, methylparathion, malathion and carbaryl, are highly toxic and teratogenic to mammals. Several chemical insecticides, such as DDT, have extremely high stability in nature. The accumulation of residual chemicals, together with notable stability pose a great problem on environmental health.

The nuclear polyhydrosis viruses (NPVs) are known as a group of viruses pathogenic to invertebrates. This group of viruses causes lethal disease in their insect hosts. The virus particles of this group usually consist of enveloped nucleocapsids which frequently are included in a large protein lattice or polyhedron. The nucleocapsids are rod-shaped with dimensions of about 250 x 50 mμ, and are usually singly enveloped; but in some instances, more than one nucleocapsid can occur within one virus envelope. Viral genomes of ths group were found to contain covalently closed supercoiled double-stranded DNA with a molecular weight of approximately 75 to 100 x 10^6 daltons (Summers and Anderson, 1973; Summers, 1977). These viruses comprise the best known insect viruses. All together, the NPVs have been found in more than 200 species of Lepidoptera, in 20 species of Hymenoptera and in 9 species of Diptera (David, 1975). They are all in the genus Baculovirus.

These bacilliform viruses replicate in the nuclei of the infected cells. During the process of infection, a substantial portion of virions is enveloped and subsequently occluded in the protein matrix of polyhedra. The intact polyhedra are not infectious in *in vitro* insect cell cultures, but they are the key ''vector'' by which virus infections are transmitted in nature. When insect larvae ingest the polyhedra, the infectious virions are released from polyhedra through the solubilization of the protein matrix of polyhedra in the alkaline environment and by enzymatic digestion in larvae gut (Harrap, 1972 and 1973). The purified NPV DNA was proved to be infectious in the insect cell cultures (Ignoffo et al., 1971).

Several Baculoviruses have been used as viral pesticides for pest control during the last 3 decades, e.g., the NPV of the Alfalfa caterpillar (*Colias eurytheme*); the NPV of cabbage looper (*Trichoplasia ni*); the NPV of the beet army worm (*Spodoptera exigua*) and NPVs isolated from sawflies for forest protection in the USA and Canada. *T. ni* was introduced to Columbia, South America from California and has been used with great success in recent years (Falcon, 1977).

Four NPV viral insecticides have been registered in the USA, and are commercially available for field application. The first, ''Elcar'', containing the NPV of the boll worm (*Heliothis zea*) is registered by the pharmaceutical firm Sandoz, Inc., for the control of cotton bollworm. The second available product named ''TM Bioctrol 1'' is registered by the US

Forest Service, and contains the NPV of the Douglas fir tussock moth (Orgyia pseudotsugata). The NPV of the Gypsy Moth (Porthetria dispar) and NPV of pine sawfly (Neodiprion sertifer) are two other NPVs which have been registered.

With the hazardous environmental deterioration by chemical pesticides, and with urgent need for promoting the world food production in mind, the use of viral pesticides might be conceptually a practical and useful approach. But before any great revolutionary events happen, a precise evaluation of the benefit as well as the potential environmental health problem exhibited by this approach should be made. It is estimated that in the western hemisphere, 30% of the current pest problems in agricultural crop production can be treated with viral pesticides (Falcon, 1977). In California among the pest species group causing major crop losses, 46% are susceptible to baculoviruses. (Martignoni, 1975). Theoretically, viral pesticides can effectively solve certain problems such as toxic chemical polution and inefficiencies of certain chemical pesticides in crop production. As far as safety and environmental health is concerned, relative amounts of in vivo and in vitro tests have been performed. But most of the tests applied used acute infectivity, antigenicity and morphological alteration as measuring factors. The fate of viral DNA, possibilities of genetic recombination and viral gene integration, viral oncogenicity as well as low level of persistent infection have never been extensively examined.

As mentioned by Tinsley and Melnick (1974), there are several important considerations and noteworthy facts to be carefully examined and evaluated. First, the candidate pesticidal virus may infect insect hosts other than the target pest. Second, insect virus may be able to induce infection in other invertebrate or even vertebrate via either permissive or abortive infection. Third, as the consequence of persistent infection or non-fetal infection, the insects are known to be carriers of a variety of animal aborviruses. Pesticidal virus might follow the same pattern, and introduce itself into human beings or other vertebrate through its vector host by an unnatural route. Fourth, the so-called host specificity in virology is neither a fundamental nor a stable characteristic; the condition of the host and the nature of infectious agent (intact virion or naked DNA) will affect the entire susceptibility to infection. Although numerous in vitro and in vivo experiments have been done to prove the species specificity and the safety of pesticide virus, the striking report of transfection of Fogh-Lund human amnion cell with the silk worm NPV-DNA (Himeno et al., 1967) and the demonstration of viral DNA and antigens in vertebrate cells (McIntosh and Kimura, 1974; Granados 1976) have raised the question of species specificity and real meaning of safety as monitored solely by the infectivity and cytopathic effect. Furthermore, various cocarcinogens and tumor promoting agents, such as phorbol ester, which probably exist widely in nature, might induce an unexpected virus and host interaction which might lead to the oncogenic transformation of cells infected by pesticidal viruses.

In the application of pesticidal viruses, two important issues require immediate attention. First of all, it is essential to improve the methodology and sensitivity in detecting virus and host cell (including vertebrate cell

and human cell) interaction at the molecular level and effects of cocarcinogen on virus and host cell interaction; the alternative way of virus infection, the fate of viral DNA, possible viral gene integration and recombination, viral oncogenicity and persistent infection require a molecular biological method of detection and observation other than infectivity assay. Secondly, the structure, function and genetic relatedness of baculovirus have to be carefully studied and examined; a universal pesticidal virus or a multifunctional pesticidal virus may be constructed.

Other than the classic methods of detection and analysis, there are several recent major technical approaches which can be applied to insect virus systems and will add a great impact to the understanding of viral genome status, gene structure, gene function and pathogenesis. Such as:

(a) Nucleic acid hybridization (including DNA-DNA reassociation kinetics analysis, in situ RNA-DNA cytohybridization, Southern's blot hybridization, etc.)

Detection of viral DNA, defective or non-defective, can be achieved by DNA-DNA reassociation kinetics analysis (Huang and Pagano, 1977). Using highly specific radioactive viral DNA probes, it has been possible to detect small numbers of copies or portions of viral genomes in the DNA isolated from cells suspected of carrying viral information. It does not matter whether viral DNA is replicating or defective, integrated or plasmid, biologically active or latent. This technique is able to tell the degree of homology and relatedness between two viruses or two individuals. The degree of viral gene expression, in regard to transcriptional mRNA, can also be detected by this technique.

As far as localization of viral nucleic acid and detection of susceptible cell types is concerned, the technique of in situ RNA-DNA cytohybridization will fulfill the goal (Huang et al., 1973, Huang and Pagano, 1977). The great advantage of this technique is its ability to localize virus-specific DNA or RNA according to cell type and intracellular location by autoradiography. In combination with these nucleic acid hybridization techniques, a more advanced study of the interaction of insecticidal virus with the mammalian cell, especially human cells, can be achieved.

(b) Restriction endonuclease and specific DNA fragmentation.

The DNA fragmentation by restriction endonuclease has become a very powerful tool for analyzing not only small viral genomes but also genomes of increasing complexity and molecular size. Cleavage of DNA into specific terminal fragments and construction of a DNA fragment map will provide elements needed for the detailed characterization of viral genome, and also for the regulation of gene transcription and gene interaction. The restriction enzyme cleavage pattern will also provide a detailed comparison of strain variation and strain relatedness.

In adenovirus system, by DNA fragment transfection and DNA-DNA reassociation kinetics analysis (using restriction endonuclease fragments as probe), it was found that only the extreme left-hand 7% of the adenovirus type 2 DNA is sufficient for transformation of rat kidney cell *in vitro* Gallimore 1974; Graham et al., 1974). The EcoR1-C fragment, the left 16% of the viral genome, of adenovirus type 12 DNA has been proved to carry a transforming gene, and was used as a powerful probe for the study of the association of adenovirus type 2 with various types of human cancer (Mackey et al., 1976).

The structure and function of several viral genomes such as ØX174, SV40, adenovirus, etc., have been elucidated by the application of restriction endonucleases. Using DNA fragments generated by various restriction enzymes and nucleic acid hybridization techniques, the virus gene expression and gene regulation in SV40 and adenovirus-infected permissive and non-permissive cells have been defined. The utilization of restriction endonuclease and nucleic acid hybridization in the human cytomegalovirus system has been very successfully performed in our laboratory. We feel that these techniques can be effectively applied to study gene interaction and gene expression in pesticidal virus-infected permissive and non-permissive cells.

(c) Transfection of viral DNA using calcium phosphate and dimethylsulfoxide (DMSO).

Viral infection can be initiated in an alternate route in an *in vitro* system. By infection of cells treated with calcium phosphate and DMSO, adenovirus DNA and herpes simplex DNA have been proved to be infectious (Graham and Van Der Eb 1973a). It is not necessary to have intact virus particles to initiate the infection process. Transformation of rat cells by DNA of adenovirus type 5 was also achieved by this method (Graham and Van Der Eb, 1973b). As mentioned above, the specific DNA fragment carrying the transforming gene has also been detected by calcium phosphate method. Using this technique to advantage, there is an urgent need for the examination of the biological activity of pesticidal viral DNA. Mass application of pesticidal virus will generate numerous defective or naked DNA and on some occasions these particles might become a potential environmental hazard and dangerous to human health.

(d) Gene cloning and recombinant DNA technology.

Gene cloning and recombinant DNA technology has become a revolutionary tool not only for the study of molecular biology but also for industrial application. Numerous genes of biochemical and genetic interest have been isolated and studied due to the achievements of recombinant DNA research. Virus genomes can be constructed and amplified *in vitro* without the natural hosts, and a wide host range, non-hazardous pesticidal virus might therefore be constructed with a minimum risk to health and environment.

This report contains three main elements which reflect the work we have performed with the support of a grant from EPA: the interaction of SfNPV with various mammalian cells *in vitro*, the genomic structure of SfNPV and cloning of SfNPV DNA (Hind III fragments) in plasmid pBR322. The details are described in the following sections.

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II. CONCLUSIONS AND RECOMMENDATIONS

Spodoptera frugiperda nuclear polyhydrosis virus DNA has been purified from polyhedra of the Ohio, North Carolina and Georgia strains. Viral DNA has been characterized for its size, electron microscopic morphology and base distribution. Viral genomes have been further studied by restriction fragmentation for genomic structure and srain variation. Viruses isolated from different geographical areas have their own identities in DNA restriction patterns.

To facilitate the molecular biological study of this virus, a DNA restriction fragment map has been made, and a library of recombinant clones carrying inserts of various viral DNA fragments have been constructed. The availability of DNA fragment map and the library of recombinant clones will make further molecular biological studies of SfNPV more fruitful.

Virus from hemolymph of SfNPV infected insects and viral DNA purified from polyhedra have been used to infect and transfect mammalian cells and insect cells from its host.

No productive infection or morphological transformation have been observed in mammalian systems, while positive infection was observed in the cell from its own host. The application of advanced nucleic acid hybridization confirmed the immunological as well as infectivity results. Based on these observations it is concluded that SfNPV does not cause severe harm to human, murine or other rodent cells *in vitro*. The extension of these studies to other insect viruses and to other mammalian cell systems is needed before there is extensive application of pesticidal viruses in the field.

Recombinant DNA research is well advanced in the field of biology in this country. It is essential for government to encourage scientists to devote time and effort on the construction of multi-variant pesticidal virus with no pathogenicity to humans and other mammals. The success in this field may alleviate the problem of toxicity created by chemical pesticides.

II. SCIENTIFIC EXPERIMENTS AND RESULTS

Section A. General Methods for Purification of S. frugiperda NPV and Viral DNA

Virus Purification. The partial purification of polyhedra of the Ohio strain of SfNPV was performed at the Southern Grain Insects Laboratory from the lysate of virus-infected Fall army worm larvae in Tifton, Ga. Subsequently, this suspension of polyhedra was washed by repeated centrifugation in a Sorvall RC-5 refrigerated centrifuge at 5,000 rpm for 5 min and resuspended in distilled water until the supernatant fluid was clear. After the last centrifugation, the polyhedra were suspended in a freshly prepared solution of 0.1M Na_2CO_3 for 15 to 20 min at room temperature and centrifuged for 5 min at 5,000 rpm. This procedure was repeated two to three times until the alkaline dissolution of the polyhedra was completed. The supernatant from each centrifugation was pooled, layered gently onto 20 to 60% (wt/wt) sucrose gradients made in TBS (0.15 M NaCl-0.05 M Tris-hydrochloride, pH 7.4) and centrifuged in an SW-27 rotor for 75 min at 25,000 rpm and 4°C. The multiple virus bands in each gradient were collected through the bottom of each tube, dialyzed against TBS overnight, and stored at 4°C. If a more concentrated virus preparation is desired, the virions may be pelleted by centrifugation and resuspended in an appropriate buffer.

Extraction of viral DNA. A concentrated suspension of gradient-purified virions was digested at 55°C for 1.5h in a proteinase K digestion mixture (1% sodium dodecyl sulfate-5 mM $CaCl_2$-1 mM EDTA-100 µg of proteinase K per ml). When pronase (1 mg/ml) was substituted for proteinase K, the digestion was carried out at 37°C.

The digested mixture was carefully layered onto preformed 10 to 30% (wt/vol) sucrose gradients in TBS containing 1 mM EDTA and centrifuged in SW-27 rotors at 18,000 rpm for 18h in a Beckman L3-40 ultracentrifuge. Fractions (1 ml) were collected through the top by pumping a 60% sucrose solution through the bottom of the tube. The DNA-containing fractions as determined by UV absorption were pooled, dialyzed against 0.05 M Tris-hydroxychloride (pH 7.4)-1 mM EDTA buffer overnight and stored at 4°C.

Alternatively, a modification of the method of Radloff et al. (1967) was used. The proteinase K-digested mixture was diluted with 0.01M Tris-hydrochloride (pH 8.0)-0.01 M EDTA buffer containing ethidium bromide at a concentration of 200 µg/ml. Cesium chloride was added to the solution until the density was 1.59 µg/ml. Centrifugation was carried out in a VI-50 rotor at 36,000 rpm for 24h in a Beckman ultracentrifuge. The DNA-containing fractions were visualized by their fluorescence in UV light and collected. The ethidium bromide was removed by extraction with isoamyl alcohol, and the DNA fractions were dialyzed against 0.05 M Tris-hydrochloride (pH 7.4)-0.001M EDTA buffer overnight and stored at 4°C or further purified by sedimentation through a sucrose gradient as previously described.

If intact DNA molecules were not required, the DNA solution could be extracted with phenol, and the DNA could be precipitated with cold ethanol at -20°C and redissolved in an appropriate buffer.

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Section B: Analysis of the *Spodoptera frugiperda* Nuclear Polyhedrosis Virus Genome by Restriction Endonucleases and Electron Microscopy

(published in J. of Virology, Vol. 44, 747-751, 1982)

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ABSTRACT

Restriction endonuclease analysis was used to differentiate between four strains of *Spodoptera frugiperda* nuclear polyhedrosis virus from different geographical areas. In addition, partial denaturation was performed, and a partial denaturation map was constructed for the Ohio strain of this virus.

EXPERIMENTAL METHODS AND RESULTS

With the increasing interest in the use of insect viruses as agents for the biological control of insect pests, there is an urgent need to identify and characterize insect viruses and various virus isolates. In this report, the restriction endonuclease patterns of DNA from a strain of *Spodoptera frugiperda* nuclear polyhedrosis virus (SfNPV) for BamHI, EcoRI, and HindIII were determined and used to differentiate between SfNPV isolates from Georgia (GA), Mississippi (MS), North Carolina (NC), and Ohio (OH). In addition, a partial denaturation map of the OH strain of SfNPV was constructed.

The strains of SfNPV were originally isolated from diseased fall armyworm larvae at Tifton, Ga.; Starkville, Miss.; Plymouth, N.C.; and Cleveland, Ohio. The virions were purified from the lysate of virus-infected fall armyworm larvae by differential centrifugation and sucrose gradients as previously described (8). The extraction of DNA from the virions, its digestion by restriction endonucleases, and the *in vitro* labeling of DNA restriction fragments and their visualization after agarose gel electrophoresis were performed essentially as described in our previous report (8).

The partial denaturation map was constructed as follows. Purified viral DNA was partially denatured by a modification of the method of Inman and Schnos (3) as described by Wadsworth et al. (11) and Kilpatrick and Huang (5). Specifically, a 10 µl sample of DNA (6 to 10 µg/ml) was mixed with an equal volume of denaturation buffer at room temperature and allowed to react for 7 min. The denaturation buffer consisted of 20% (vol/vol) formaldehyde, 0.02 M Na_2CO_3, 5 mM EDTA, and enough NaOH to bring the pH up to an appropriate value. It was found empirically that a pH of 11.15 gave the most distinct partial denaturation pattern, and denaturation was already quite extensive at

pH 11.25. The reaction was stopped by the addition of 80 µl of ice-cold spreading solution consisting of 70 µl of 1M ammonium acetate, 5 µl of 0.2M acetic acid, and 5 µl of cytochrome c (2 mg/ml) per 20 µl of the denatured DNA solution. The pH of the final solution was about 5.2.

The aqueous method (6) of spreading partially denatured DNA molecules (5,11) was used to prepare the specimen grids. Immediately after the termination of partial denaturation, 1 µl each of denatured and completely alkaline-denatured ØX174 RF molecules were added to the reaction mixture as internal length standards. A 50 µl amount of this solution was spread over the surface of an 0.3 M ammonium acetate solution adjusted to pH 5.2. The DNA-cytochrome c film was immediately transferred to parlodion-coated, 200-mesh copper grids by surface contact, stained with uranyl acetate, dehydrated in 90% ethanol, rotary shadowed with platinum-palladium (80:20) alloy, and stabilized with a carbon coating to minimize distortions from the electron beam.

The sample grids were examined in a Hitachi H-500 electron microscope at 50 kV. The electron micrographs of DNA molecules were taken at magnifications ranging from 3,000 to 9,000. The micrographs were enlarged by an overhead projector, and only intact, circular, relatively untangled DNA molecules were used for length measurements. A programmed Hewlett-Packard 9825A calculator and digitizer was used to trace the projected DNA molecules, and lengths were recorded in microns. ØX174 RF DNA, with a known molecular weight of 3.48×10^6 (10), was used as a standard. For partially denatured molecules, the lengths of the single-stranded and double-stranded regions were measured separately. The lengths of the single-stranded regions were then corrected for shrinkage by a factor of 1.418, a value obtained empirically by comparing the molecular lengths of alkaline-denatured and intact ØX174 RF DNA molecules cospread with the partially denatured SfNPV DNA molecules.

The OH strain of SfNPV was chosen for detailed analysis. The buoyant density of the viral DNA was found to be 1.6992 ± 0.0003 g/ml by equilibrium CsCl gradient centrifugation in a Spinco model E analytical ultracentrifuge, with *Micrococcus lysodeikticus* DNA used as a density marker (ρ=1.731 g/ml). Thus, the viral DNA should have an average guanine plus cytosine (G+C) content of 40% as calculated by the equation derived by Schildkraut et al. (9). The molecular weight of the viral genome was found to be $8.25 (\pm 5.2) \times 10^6$ by electron microscopy. By these parameters, it was virtually indistinguishable from the genome of the GA strain of SfNPV, from which the SfNPV strains propagated in most other laboratories in the United States and Europe were originally derived. The molecular weight and density data for SfNPV DNA obtained in our laboratory agree reasonably well with the values reported previously (1,2,4,7).

The SfNPV OH genome was cleaved into 8, 15, and 25 fragments by the restriction endonucleases BamHI, HindIII, and EcoRI, respectively (Fig. 1). The molecular weights of these restriction fragments and their designations were reported in a previous paper (8).

Viral DNA from the GA, MS, OH, and NC strains SfNPV were cleaved with BamHI, HindIII, or EcoRI, end-labeled, and electrophoretically separated on 0.7% agarose gels. The resulting autoradiographs are shown in Fig. 2. The migration patterns of the HindIII digests were identical for the MS, NC, and OH strains. The extra fragment present in the GA srain may be due to heterogeneity within the virus preparation. The EcoR1 digests of the GA and MS strains had migration patterns that were easily distinguishable from those of the NC and OH strains. Heterogeneity may account for the presence of some of the submolar fragments observed. Loss of EcoR1 sites, possibly between some of the linked comigrating fragments such as EcoR1 fragments C and D, may also explain the appearance of extra high-molecular-weight restriction fragments (e.g., the EcoR1 fragment above EcoR1-A in the MS digest [Fig. 1C and 2]).

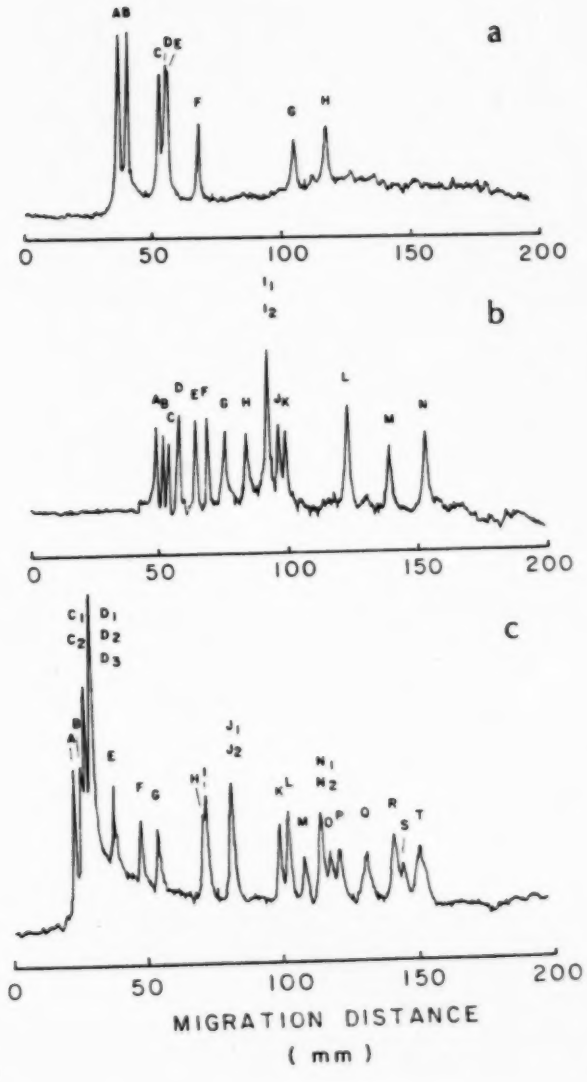

Fig. 1. Microdensitometer scans of autoradiographs of electrophoretically separated, end-labeled SfNPV OH DNA cleaved by (a) BamHI, (b) HindIII, or (c) EcoR1. Fragment S in the EcoR1 digest is the only fragment present in submolar (0.5 mol) amounts. All cleavage patterns were scanned at a 1:1 scan-to-record ratio.

Virions usd for DNA purification were purified from the lysate of virus-infected larvae cloned *in vivo*. *In vitro* plaque purification of the various virus strains was not done because of the lack of a sound permissive cell system which can generate infectious virus in SfNPV-infected cell cultures. Therefore, confirmation of the loss of a specific restriction enzyme site must await DNA sequencing data and the construction of a complete restriction map of the viral genome of EcoR1. On the other hand, the migration patterns of the BamHI digests were quite distinct for each of the four strains of SfNPV. The fact that SfNPV OH had the largest number of BamHI sites was one reason this strain was chosen for detailed analysis and restriction mapping in our laboratory. From the known restriction map for BamHI (8) and the sizes of the BamHI restriction fragments, we can deduce that the NC strain might have lost the BamHI site between BamHI fragments A and G; the MS strain has lost BamHII fragments A and D, and the GA strain has lost the BamHI site between BamHI fragments A and D (Fig. 1 and 2). Again, a final conclusion about the loss of BamHI sites in these cases can only be made with the support of DNA sequencing data.

Preliminary experiments showed that the nick-translated BamHI-G and D/E fragments eluted from gels of a BamHI digest of SfNPV OH DNA did hybridize to the largest BamHI restriction fragment of the other strains, as predicted.

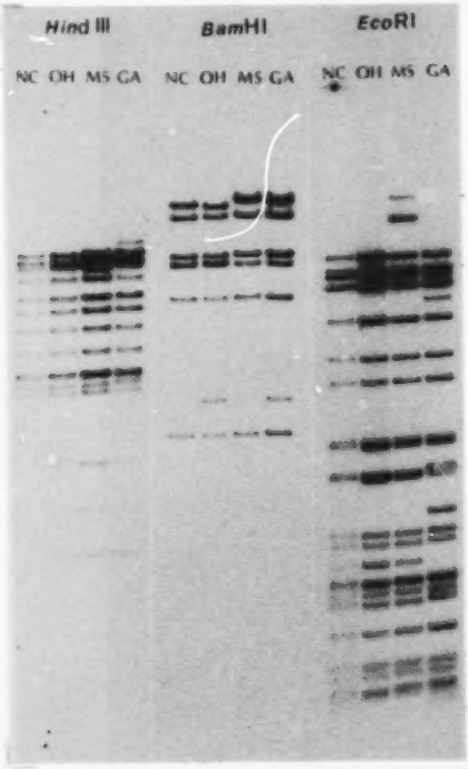

Fig 2. Cleavage patterns of DNAs from the NC, OH, MS, and GA strains of SfNPV with restriction endonucleases BamHI, HindIII, and EcoRI. The restriction fragments were end-labeled with [α-^{32}P]dATP and electrophoretically separated in an 0.7% agarose gel.

To provide a means for orienting the circular viral DNA molecule, we constructed a partial denaturation map for the SfNPV OH genome. Partial denaturation of SfNPV DNA was initially performed at pH values ranging from 11.0 to 11.6, the reaction time being fixed at 7 min at room temperature (25°C). Small denatured regions were detectable as tiny ''bubbles'' along circular molecules at pH 11.15 (Fig. 3), but denaturation became extensive at pH 11.25. When the pH was raised above 11.25, extensive single-stranded regions were seen throughout the circular molecule, and distinct patterns were no longer discernible. Thus, it was not feasible to construct a precise denaturation map of the SfNPV genome because of the lack of a restriction enzyme which cleaves this DNA molecule at only one site. Therefore, the partial denaturation map of the SfNPV genome presented here was constructed from data obtained by denaturation at pH 11.15 and supplemented by data from denaturation at pH 11.25 by arbitrarily setting the major adenine plus thymine (A+T)-rich region of the molecule as the origin of the partial denaturation map during the alignment procedure described below.

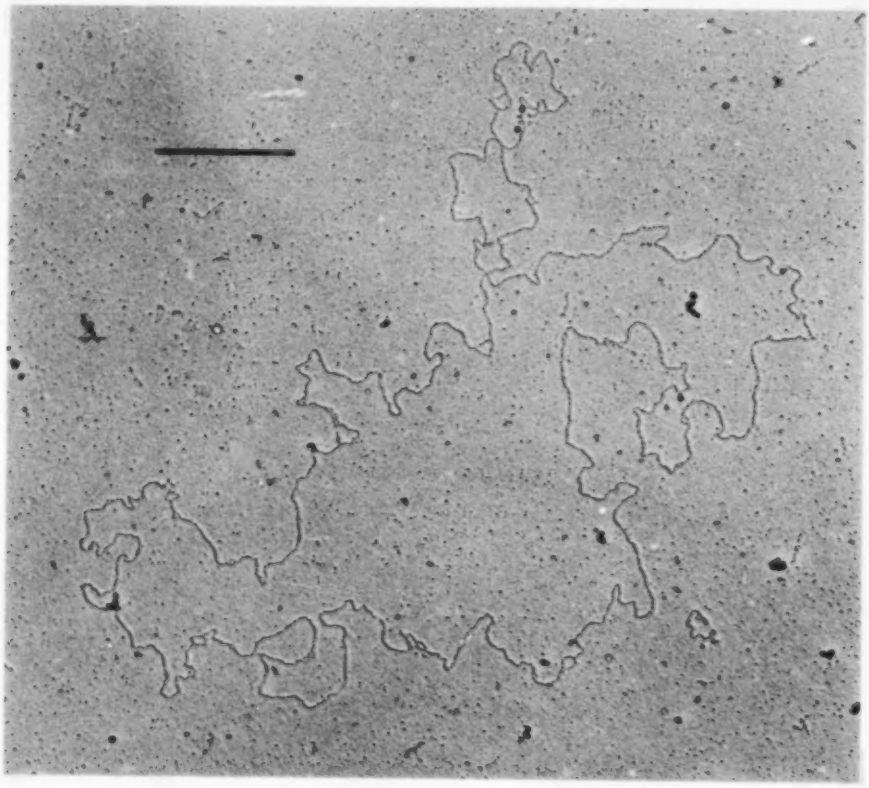

Fig. 3. Electron micrograph of a partially denatured SfNPV OH DNA molecule. Partial denaturation was accomplished in alkaline formaldehyde at pH 11.15 for 7 min at 25°C, and the DNA was spread by an aqueous method as described in the text. The small bubbles represent the denatured single-stranded region. Bar, 1 μm.

We facilitated the data analysis by photographing only relaxed circular DNA molecules for length measurements. Because of the slight length variations between different DNA spreads, it was decided that the best way to compare the data from different experiments was to express all single- and double-stranded lengths as a percentage of total circular length instead of as absolute length units. It was found that with few exceptions, after correction for single-stranded shrinkage, the total circular lengths of partially denatured molecules were comparable to those of undenatured DNA molecules spread under similar conditions as controls. This justified the use of the shrinkage factor of 1.418 described above.

During the alignment of the partially denatured molecules, representations of these DNA molecules were plotted on strips of graph paper on a scale of 10% of the total length per inch. The molecules were then arranged for maximum overlap between the few A+T-rich and G+C-rich regions. The frequency of occurrence of the denaturation sites along the DNA of 14 molecules examined was then calculated to give the tentative partial denaturation map (Fig. 4).

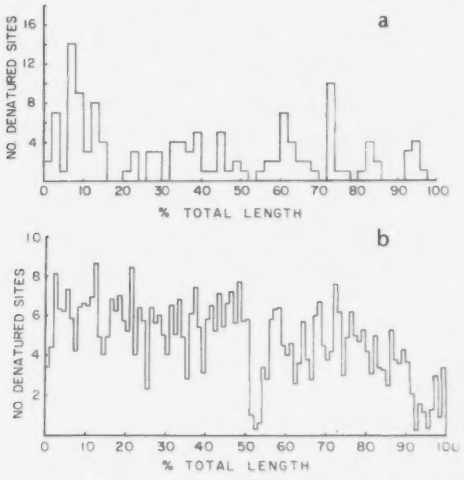

Fig. 4. Histograms showing the positions and frequencies of denatured sites for SfNPV OH DNA after partial denaturation at (a) pH 1.15 or (b) pH 11.25. The Y axis represents the number of denatured sites per 2% of the total length (a) or the number of denatured sites per 1% of the total length (b).

Beginning from the origin of the map (Fig. 4a), there was a major relative A+T-rich zone which extended for about 15% of the total length. There followed a region of lower A+T content that stretched for about 30% of the molecule; at pH 11.25 (Fig. 4b) most of this region was denatured, but at 11.15 there were a few small, relatively G+C-rich sites scattered around this region. The first G+C zone was found to be located immediately next to the central portion of the map and could only be recognized at pH 11.25. Another G+C-rich zone spanned the terminal 15% of the map. These two regions were the only ones that remained undenatured at pH 11.25. The region between them was marked by two relative A+T-rich sites.

In summary, we characterized four geographically different strains of SfNPV by restriction endonuclease digestion. In addition, a partial denaturation map of the SfNPV OH genome was constructed. There was no indication of the presence of long stretches of high G+C or high A+T regions or of highly repetitive genome sequences, as was the case with certain herpesviruses. However, the asymmetrical pattern of the two G+C-rich regions shown in the denaturation map at pH 11.25 might provide a means for orienting the circular viral DNA molecule.

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Section C: *Spodoptera frugiperda* Nuclear Polyhedrosis Virus Genome: Physical Maps for Restriction Endonucleases BamHI and HindIII

(published in J. of Virology, Vol. 38: 922-931, 1981)

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ABSTRACT

The physical map for the genome of *Spodoptera frugiperda* nuclear polyhedrosis virus was constructed for restriction endonucleases BamHI and HindIII. The ordering of the restriction fragments was accomplished by cross-blot hybridization of BamHI, HindIII, and EcoR1 fragments. The alignment of the HindIII fragments within the BamHI map was achieved by double digestion with the two restriction endonucleases followed by cross-blot hybridization. The results showed that the viral genome consisted of mainly unique sequences. In addition, the circular nature of the viral genome was reaffirmed.

INTRODUCTION

Spodoptera frugiperda nuclear polyhedrosis virus (SfNPV) is a member of the baculovirus group with a double-stranded, circular, supercoiled DNA genome (16). The molecular weight of the DNA molecule has been determined to be about 80 x 10^6 by electron microscopy and restriction endonuclease analysis in this laboratory (J. Virol. 44: 747-751, 1982), in reasonable agreement with previous reports by other investigators (1,2,4,7,8,16).

Because of its relatively large size and complexity, the construction of a restriction map of the viral genome should greatly facilitate the study of its molecular biology. It would allow investigators to study virus recombinants and map the crossover sites (14,17). The comparison of different virus strains by restriction endonuclease analysis (9,13) is more meaningful when it is possible to locate the regions showing the greatest genetic variation between virus strains. Even more important, it is essential for the study of gene transcription and regulation in both permissive and non-permissive cells. In this paper, a physical map of the SfNPV genome was constructed with the restriction endonucleases BamHI and HindIII.

MATERIALS AND METHODS

Virus purification. The partial purification of polyhedra of tthe Ohio strain of SfNPV was performed at the Southern Grain Insects Laboratory from the lysate of virus-infected Fall army worm larvae in Tifton, Ga. Subsequently, this suspension of polyhedra was washed by repeated

centrifugation in a Sorvall RC-5 refrigerated centrifuge at 5,000 rpm for 5 min and resuspension in distilled water until the supernatant fluid was clear. After the last centrifugationn, the polyhedra were suspended in a freshly prepared solution of 0.1 M Na_2CO_3 for 15 to 20 min at room temperature and centrifuged foor 5 min at 5,000 rpm. This procedure was repeated two to three times until the alkaline dissolution of the polyhedra was complete. The supernatant from each centrifugation was pooled, layered gently onto 20 to 60% (wt/wt) sucrose gradients made in TBS (0.15 M NaCl-0.05M Tris-hydrochloride, pH 7.4) and centrifuged in a SW-27 rotor for 75 min at 25,000 rpm and 4°C. The multiple virus bands in each gradient were collected through the bottom of each tube, dialyzed against TBS overnight, and stored at 4°C. If a more concentrated virus preparation is desired, the virions may be pelleted by centrifugation and resuspended in an appropriate buffer.

Extraction of viral DNA. A concentrated suspension of gradient-purified virions was digested at 55°C for 1.5 h in a proteinase K digestion mixture (1% sodium dodecyl sulfate-5 mM $CaCl_2$-1 mM EDTA-100 µg of proteinase K per ml). When pronase (1 mg/ml) was substituted for proteinase K, the digestion was carried out at 37°C.

The digested mixture was carefully layered onto preformed 10 to 30% (wt/vol) sucrose gradients in TBS containing 1 mM EDTA and centrifuged in SW-27 rotors at 18,000 rpm for 18 h in a Beckman L3-40 ultracentrifuge. Fractions (1 ml) were collected through the top by pumping a 60% sucrose solution through the bottom of the tube. The DNA-containing fractions as determined by UV absorption were pooled, dialyzed against 0.05 M Tris-hydrochloride (pH 7.4)-1 mM EDTA buffer overnight and stored at 4°C.

Alternatively, a modification of the method of Radloff et al. (10) was used. The proteinase K-digested mixture was diluted with 0.01 M Tris-hydrochloride (pH 810)-0.01 M EDTA buffer containing ethidium bromide at a concentration of 200 µg/ml. Cesium chloride was added to the solution until the density was 1.59 g/ml. Centrifugation was carried out in a VI-50 rotor at 36,000 rpm for 24 h in a Beckman ultracentrifuge. The DNA-containing fractions were visualized by their fluorescence in UV light and collected. The ethidium bromide was removed by extraction with isoamyl alcohol, and the DNA fractions were dialyzed against 0.05 M Tris-hydrochloride (pH 7.4)-0.001 M EDTA buffer overnight and stored at 4°C or further purified by sedimentation through a sucrose gradient as previously described.

If intact DNA molecules were not required, the DNA solution could be extracted with phenol, and the DNA could be precipitated with cold ethanol at -20°C and redissolved in an appropriate buffer.

Restriction enzyme digestion of viral DNA. Purified viral DNA was digested with EcoR1, BamHI, or HindIII in a solution containing 0.05 M Tris-hydrochloride (pH 7.4)-0.01 M $MgCl_2$-0.10 M NaCl-0.006 M β-mercaptoethanol. Incubations were generally for 3 h at 37°C, and sufficient enzyme was added for complete digestion within this period. When partial digestion of the viral DNA became necessary for mapping purposes, the same amount of restriction enzyme was added, but incubation was carried out at

10°C for between 10 and 30 min. The reaction was stopped by adding EDTA to a final concentration of 10 mM. The restriction enzyme was then heat inactivated at 70°C for 20 min.

The restriction enzyme EcoR1 was purified from Escherichia coli strain Ry13 (3), HindIII was purchased from Bethesda Research Laboratories, Inc., Rockville, Md., and BamHI was purified from Bacillus amyloliquefaciens H (19; L.A. Smith and J.G. Chirikjian, Fed. Proc. 36: 908, 1977).

In vitro labeling of DNA. For critical determinations of the molecular weights and stoichiometry of DNA fragments by electrophoresis in agarose gels, the DNA restriction fragments were end labeled in vitro with avian myeloblastosis virus reverse transcriptase obtained from G.E. Houts of the Life Sciences Institute, St. Petersburg, Fla. Approximately 10 μCi of [α-^{32}P]dATP (450 Ci/mmol; ICN Pharmaceuticals) were lyophilized in a small tube and redissolved in a reaction mixture containing 20 to 25 μg of DNA restriction fragments per ml, 100 mM and NaCl, 50 mM Tris-hydrochloride (pH 7.4), 6 mM β-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mM dCTP, 0.1 mM dGTP, and 0.1 mM dTTP. Reverse transcriptase (15 U) was added, and incubation was carried out at 4°C for 20 to 30 min. The reaction was stopped by heat inactivating the enzyme in a 70°C water bath for 20 min. The mixture was then treated with proteinase K (50 μg/ml) for 30 min at 37°C before gel electrophoresis.

For cross-blot hybridization experiments, labeling was done with DNA polymerase I instead of reverse transcriptase. The procedure, a modification of the method of Rigby et al. (12), was essentially the same as that described in the preceding paragraph, except that 5 μl of DNA polymerase I was used, the $MnCl_2$ was not needed, and incubation was carried out at 14°C for 30 min. It was found that this procedure can be adopted for end labeling DNA restriction fragments as well. The DNA polymerase I used in these experiments was purified by the method of Richardson et al. (11) as modified by Jovin et al. (6).

Agarose gel electrophoresis of DNA fragments. The DNA restriction fragments were fractionated by electrophoresis through 0.7% agarose (SeaKem) gels. Agarose was dissolved in E buffer (0.04 M Tris-hydrochloride-0.02 M sodium acetate-1 mM EDTA, pH 7.2) and allowed to solidify on a glass plate to form a horizontal slab gel (6 by 200 by 250 mm). For cross-blot hybridization experiments, slightly wider gels (6 by 250 by 250 mm) were used. Sample slots of suitable sizes were formed by inserting a plexiglas comb at one end of the gel before gel formation was complete. DNA samples were loaded into the slots with tracer amounts of a bromophenol blue solution made up in E buffer with 30% glycerol.

The DNA was electrophoresed at a constant voltage of 100 V at 25°C for about 18 h until the bromophenol blue marker reached the end of the gel.

Visualization of DNA fragments. When unlabeled DNA samples were electrophoresed, the gel was stained with ethidium bromide (20 μg/ml in E buffer) for 1hr, and the DNA fragments could be visualized under UV light and photographed with a Polaroid MP-4 camera with an orange filter under the lens.

In the case of labeled DNA samples, the gel was dehydrated by vacuum on a gel dryer and exposed to Kodak X-Omat R X-ray film for 24 to 48 h. The positions of the DNA fragments were thus recorded on the developed film. For molecular weight measurements, lambda DNA digested with EcoR1, BamHI, or HindIII was used for molecular weight markers. The migration distances of the DNA fragments as recorded on film were determined by a Hewlett Packard 9825A calculator and digitizer programmed to calculate the DNA fragment sizes in kilobases when suitable molecular weight markers were provided. Densitometer tracings of the autoradiographs were done with a Joyce Loebl microdensitometer. The molar ratios of the DNA fragments, being proportional to the areas under the peaks, were then determined by cutting out and weighing the corresponding peaks on these tracings.

Cross-blot hybridization. To detect homology between different fragments, the Hutchison cross-blot hybridization technique (C. Hutchison, personal communication) was used. A ''cold'' restriction digest was run across the entire width of a square slab gel (6 by 250 by 250 mm) using 10 to 15 μg of viral DNA per gel. The positions of the DNA bands could be photographed under UV light after staining with ethidium bromide. The DNA in the gel was then depurinated partially with 0.25 M NaOH-1 M NaCl, and neutralized in 1 M Tris-hydrochloride (pH 7.4)-1.5 M NaCl to improve the efficiency of transfer of large DNA fragments onto a nitrocellulose sheet (18). The actual transfer was done in 6 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 8 h by the procedure of Southern (15) with slight modifications. The nitrocellulose sheet (Bethesda Research Laboratories) was then air dried briefly and baked in vacuum at 80°C for 3 h.

A ^{32}P-labeled restriction digest was run and prepared for transfer as described above. The nitrocellulose sheet with the immobilized DNA was placed on top of the radioactive gel such that each cold band bound to the nitrocellulose sheet intersected each ''hot'' band in the gel at right angles. The hot band patterns were then transferred and directly hybridized at right angles to the unlabeled DNA bands on the nitrocellulose sheet in hybridization buffer (6 x SSC-0.1% sodium dodecyl sulfate) at 65°C before the transfer and hybridization step. Finally, the nitrocellulose sheet was washed as described by Jeffreys and Flavell (5), blotted dry, wrapped in a protective plastic freezer bag, and exposed to Kodak O-Omat R X-ray film for 3 days. A DuPont Lightning-Plus intensifier screen was often used to shorten the exposure time. After development, spots of developed silver grains should mark the intersection points of DNA fragments sharing common sequences.

RESULTS

Restriction enzyme cleavage patterns. The SF NPV genome was cleaved into 8, 15, and 25 fragments by the restriction enzymes BamHI, HindIII, and EcoR1, respectively (Fig. 1). The estimated molecular weights of the restriction fragments (see Table 6) have already been reported (manuscript submitted). As described in a later section, slight corrections to the molecular weights of BamHI fragments were made. It is seen that there are

four sets of comigrating fragments in the EcoRI digest, one in the HindIII digest, and none in the BamHI digest. Thus, it was decided that the construction of restriction maps for HindIII and BamHI should be attempted initially because they would allow less ambiguous assignment of transcription patterns and regions of genetic variation between virus strains by using the Southern blot hybridization technique.

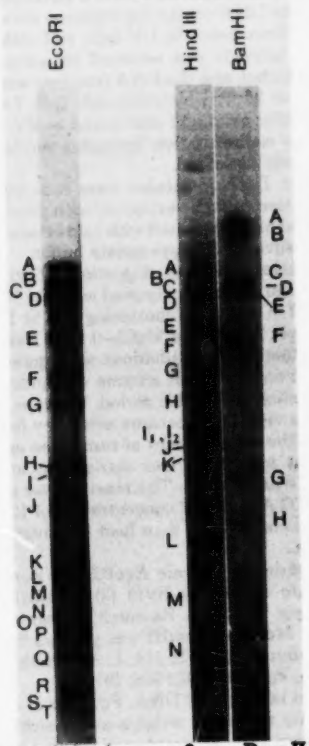

Fig. 1. Cleavage patterns of SfNPV DNA by the restriction endonucleases BamHI, HindIII, and EcoRI. The restriction fragments were labeled with [α-^{32}P]dATP in the presence of DNA polymerase I. The partial fragment between fragments D and E in the EcoRI digest was the result of slightly incomplete digestion.

Construction of a BamHI restriction map by cross-blot hybridization. The first step in the mapping procedure was to determine whether there were repeat sequences within the viral genome which could complicate the interpretation of hybridization data. This was accomplished by the cross-blot hybridization of cold BamHI fragments to ^{32}P-labeled BamHI fragments (Fig. 2). Spots were found along the diagonal only. Thus, there were probably no long repeat sequences present in the viral genome.

Partial digestion of SfNPV DNA should produce DNA fragments consisting of two or more ''complete'' restriction fragments linked together. By hybridizing ^{32}P-labeled BamHI fragments to unlabeled, partially digested BamHI fragments immobilized on a nitrocellulose sheet, the linkages between different BamHI restriction fragments could be deduced (Fig. 3 and Table 1). The deduction of the single linkages from fragments 3, 4, 5, and 6 are quite obvious. Fragment 2 has four spots, indicating sequence homologies with BamHI fragments B, E, F, and G. One of the two comigrating fragments must be fragment B, because one spot is on the diagonal where the labeled and unlabeled B fragments should intersect. Thus, the three BamHI fragments E, F, and G must be linked together to form the other comigrating fragment. Since

fragment E is directly linked to both fragments F and G, the three fragments must be linked in the manner indicated in Table 1, i.e., F-E-G. To sum up, the data in Table 1 demonstrate linkages between five BamHI fragments, and the deduced ordering is H-C-F-E-G.

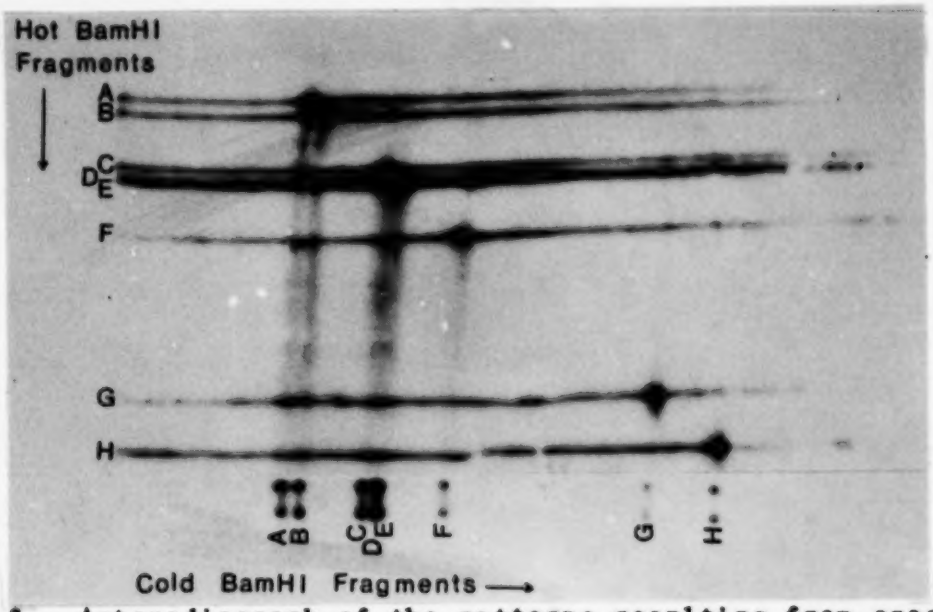

Fig. 2. Autoradiograph of the patterns resulting from cross-blot hybridization between ^{32}P-labeled and cold BamHI fragments of SF NPV DNA. A ^{32}P-labeled BamHI digest was run side by side with the cold BamHI digest and transferred to the nitrocellulose sheet. The identification of the cold BamHI fragments was thus facilitated by noting the position of the corresponding transferred ^{32}P-labeled fragment.

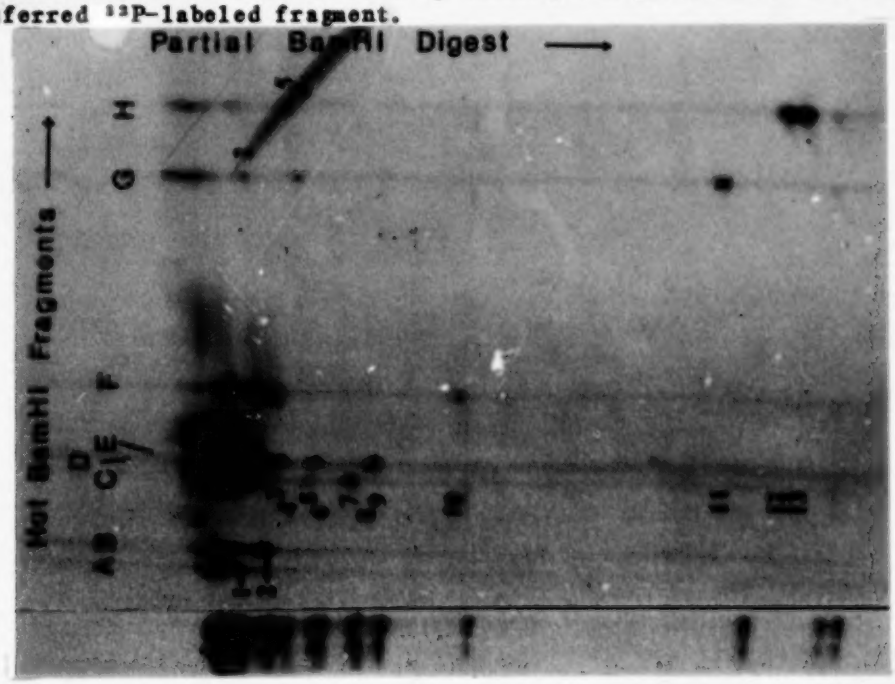

Fig. 3. Autoradiograph of the patterns resulting from the cross-blot hybridization between ^{32}P-labeled BamHI fragments and a cold partial BamHI digest of SfNPV DNA. The ^{32}P-labeled fragments of the partial digest was run side by side with the cold fragments and transferred to the nitrocellulose sheet to facilitate the identification of the unlabeled fragments.

To complete the ordering of BamHI restriction fragments, ^{32}P-labeled HindIII fragments were hybridized to unlabeled BamHI fragments in another cross-blot hybridization experiment (Fig. 4 and Table 2). If a HindIII fragment shares sequence homology with only one BamHI fragment as is the case with fragments E, G, I_1, J, K, L, M, and N, the HindIII fragment in question must be entirely within the BamHI fragment involved. However, when a HindIII fragment shares sequence homology with two or more BamHI fragments, these BamHI fragments must be linked together with the BamHI restriction sites at the linkage points lying within the HindIII fragment involved. This is illustrated in Table 2 by the HindIII fragments A, B, C, D, F, H, and I_2. Thus, with BamHI fragment B linked to fragments H and D and BamHI fragment A linked to fragments D and G, the ordering of the BamHI restriction fragments could be completed (Fig. 5a).

Construction of a HindIII restriction map by cross-blot hybridization. Using the data presented in Table 2 in combination with the known ordering of the BamHI restriction fragments, a partial ordering of the HindIII fragments could be deduced (Fig. 5b). Where uncertainties in ordering existed, the fragments involved were included in one block. This is true for HindIII fragments E, G, and L, which lie totally within BamHI fragment A, HindIII fragments I_1, and M, which lie within BamHI fragment D, and HindIII fragments K and N, which lie within BamHI fragment C.

To clear up these ambiguities, ^{32}P-labeled EcoR1 fragments were hybridized to unlabelled HindIII fragments in a cross-blot hybridization experiment (Fig. 6 and Table 3). The linkage groups were deduced by the reasoning described above. In the assignment of linkages deduced from comigrating EcoR1 fragments C_1 and C_2, it is seen that HindIII fragments D and E must be linked because fragments H and I_2 are known to be linked and located well away from both D and E. However, the assignments involving EcoR1 comigrating fragments D_1, D_2, and D_3 are more speculative. Fortunately, the ordering of the HindIII fragments involved is already precisely known. With the additional information in Table 3, the HindIII restriction map can now be deduced. It is seen that HindIII fragment E is linked to both fragments D and L. Thus, it is easy to reason that th fragments D, E, F, G, and L are joined in the order D-E-L-G-F. Similarly, knowing that fragment I_1 is linked to both fragments A and M, one can deduce that the fragments A, F, I_1, and M are linked in the order F-M-I_1-A. Finally, with HindIII fragment K linked to fragment B, the fragments B, I_2, K and N can be unambiguously joined as I_2-N-K-B. The resulting restriction map for HindIII is shown in Fig. 5c.

Double digestion of SfNPV DNA by BamHI and HindIII. The double digestion of SfNPV DNA by BamHI and HindIII serves a threefold purpose. First, it provides an independent confirmation of the restriction map deduced from cross-blot hybridization. Second, it allows the precise alignment of HindIII restriction fragments within the BamHI map or vice versa. Last, it gives better estimates of the molecular weights of large DNA fragments because these fragments are cleaved by the second enzyme into smaller fragments that are within the range of molecular weights provided by the markers. In our case, this is applicable to the BamHI fragments A and B.

TABLE 1. *Cross-blot hybridization of ^{32}P-labeled BamHI fragments to unlabeled BamHI partial digestion products*

Partial digestion products	*Bam*HI fragments with homologous sequences	Linkage groups
1	A	
2[a]	B	
	E, F, G	F-E-G
3	C, F	C-F
4	E, F	F-E
5	C, H	H-C
6	E, G	E-G
7	C	
8	D	
9	E	
10	F	
11	G	
12	H[b]	
13	H	

[a] Two comigrating fragments.

[b] Fragment 12 probably corresponds to *Bam*HI fragment H linked to a short fragment since fragment 13 is on the diagonal marking the intersection points of identical cold and ^{32}P-labeled *Bam*HI fragments. The short fragment was not observed in the 0.7% agarose gels because its small size allowed it to migrate ahead of the bromophenol blue marker.

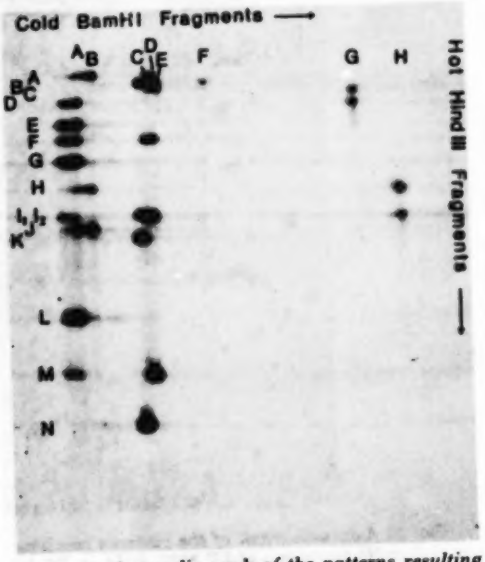

FIG. 4. *Autoradiograph of the patterns resulting from the cross-blot hybridization between ^{32}P-labeled HindIII fragments and cold BamHI fragments of SF NPV DNA. The smearing of the spots was due to the slight overloading of the sample slots during gel electrophoresis of the cold BamHI fragments.*

TABLE 2. *Cross-blot hybridization of ^{32}P-labeled HindIII fragments to unlabeled BamHI fragments*

*Hin*dIII fragment	*Bam*HI fragments with homologous sequences	Linkage groups for *Bam*HI
A	B, D	D-B
B	C, E, F	C-F-E
C	E, G	E-G
D	A, G	G-A
E	A	
F	A, D	A-D
G	A	
H	B, H	B-H
I_1	A[a], C, D, H	D
I_2		H-C
J	B	
K	C	
L	A	
M	A[a], D	
N	C	

[a] These two spots suggest a possible sequence homology between parts of *Bam*HI fragment D and *Bam*HI fragment A. The relevant sequences within *Bam*HI fragment D are located near the junction between *Hin*dIII fragments M and I_1. The sequence within *Bam*HI fragment A is located within *Hin*dIII fragment L (Fig. 8 and Table 5).

TABLE 3. *Cross-blot hybridization of ^{32}P-labeled EcoRI fragments to unlabeled HindIII fragments*

*Eco*RI fragment	*Hin*dIII fragments with homologous sequences	Linkage groups for *Hin*dIII
A	F, G, L	L-G-F
B	A, I	I_1-A
C_1	D, E, H, I	D-E, H-I_2
C_2		
D_1	B, C, D, E	B-C, C-D, E
D_2		
D_3		
E	A	
F	C	
G	I, K, N	I_2-N-K
H	F	
I	B	
J_1	C, J	
J_2		
K	M	
L	E, L	E-L
M	A	
N_1	F, K	
N_2		
O	H, J	J-H
P	F	
Q	A, J	A-J
R	B, K	K-B
S	B	
T	M, I	M-I_1

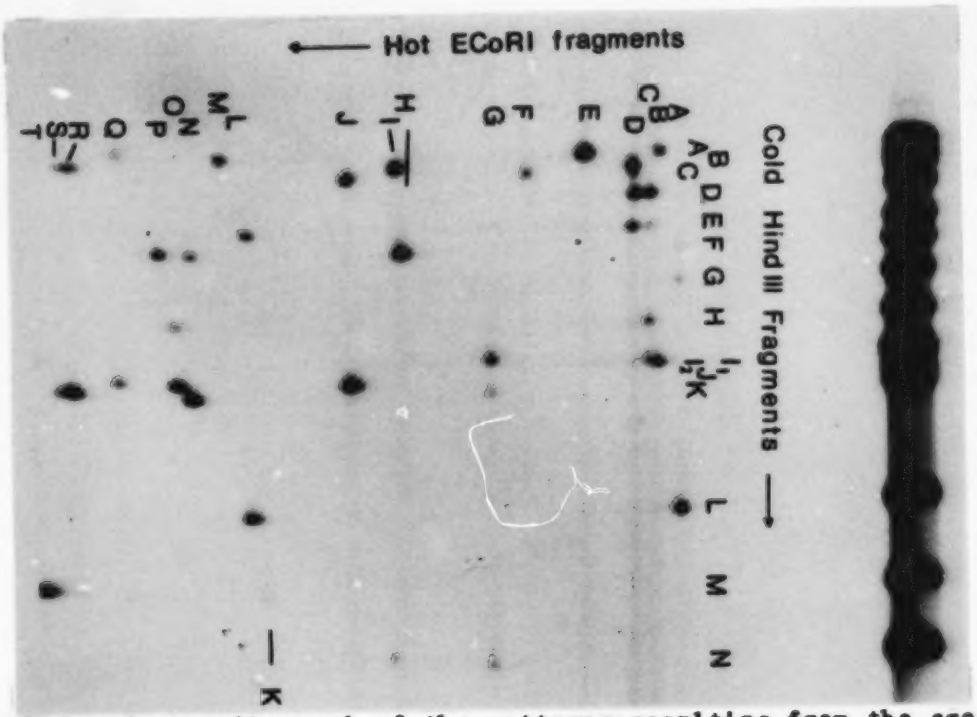

Fig. 6. Autoradiograph of the patterns resulting from the cross-blot hybridization between ^{32}P-labeled EcoRI fragments and cold HindIII fragments of SfNPV DNA.

SF NPV DNA was first digested with HindIII and then with BamHI, end labeled with [α-^{32}P]dATP, and electrophoresed in a 0.7% agarose gel. A complete HindIII digest was run at the same time so that the fragments that were cleaved by BamHI could be identified. The molecular weights and stoichiometry of the restriction fragments were estimated as described above. The BamHI and HindIII double-digest patterns are shown in Fig. 7. It is seen that the HindIII fragments B, C, D, F, H, and I_2 have disappeared. In addition, 13 new fragments are present. Referring to the restriction maps in Fig. 5, this is exactly what is expected, with HindIII fragments C, D, F, H, and I_2 each being cleaved once and fragment B cleaved twice. Fragment A should also be cleaved once. This is confirmed by its slight shift in position to one corresponding to a lower molecular weight. The cleaved-off piece of DNA is probably so short that it is running ahead of the bromophenol blue marker and his migrated out of the gel. The estimated molecular weights of these double-digestion products are presented in Table 4.

Alignment of HindIII fragments within the BamHI map. To achieve the alignment of HindIII fragmgments within the BamHI map, ^{32}P-labeled HindIII fragments were hybridized to unlabeled DNA fragments from a HindIII-BamHI double digestion in a cross-blot hybridization experiment (Fig. 8). The ^{32}P-labeled HindIII fragments will hybridize to the double-digest fragments that share their sequence homology, thus allowing the identification of specific double-digest fragments that form the original HindIII restriction fragments (Table 5). The location of BamHI sites within the HindIII fragments can then be calculated from the estimated molecular weights of the

double-digestion fragments listed in Table 4. The alignment of HindIII fragments within the BamHI map was thus accomplished. For example, the HindIII fragment D, made up of fragments 4 and 18 of the double digest, spans the BamHI fragments A and G. Since fragment 4 is too long to fit into BamHI fragment G, it must be within BamHI fragment A, whereas fragment 18 lies within BamHI fragment G. Similar deductions can be made, and the results are summarized in the third column of Table 5.

During the initial alignment, it was found that the previously determined molecular weights of BamHI fragments A and B were too low. Thus, using the estimated molecular weights of the double-digestion fragments (Table 4), more accurate values of 24.1 x 10^6 and 18.5 x 10^6, respectively, were assigned to the BamHI fragments A and B and used in the construction of the final restriction map of BamHI. The molecular weights of the other BamHI fragments were proportionally adjusted so that the size of the whole DNA molecule was again 82.5 x 10^6. The molecular weights of the HindIII and BamHI restriction fragments used in the map are presented in Table 6. The restriction map of SF NPV DNA for BamHI and HindIII is shown in Fig. 9.

DISCUSSION

The restriction map of the SF NPV DNA genome is presented in Fig. 9 in linear rather than circular form for the sake of simplicity. However, the restriction enzyme mapping data confirm the circular nature of the DNA molecule. The SF NPV genome consists of mainly unique sequences. This is in agreement with the results from Kelly's reassociation kinetics experiments (7) and our previous partial denaturation mapping data (J. Virol. 44: 747-751, 1982). Our cross-blot hybridization data (Fig. 4 and 8, Tables 2 and 5) indicate that the only possible repeat sequences of any appreciable length may be those located in HindIII fragment L and those near the junction of HindIII fragments M and I_1. However, one cannot exclude the possibility of additional short repeat sequences within parts of the genome where no BamHI, HindIII, or EcoR1 restriction sites are present.

The restriction maps for BamHI and HindIII sites should provide sufficient sectioning of the SF NPV genome to facilitate initial studies of transcription patterns and genetic variations between virus strains. The relatively precise locations of the BamHI and HindIII restriction sites are confirmed by our double-digestion data where good placement of the sites for the second enzyme within the sites of the first enzyme is possible. If a specific region of the genome needs to be studied more closely, restriction mapping with a third our fourth enzyme such as EcoR1 or XbaI may be attempted to provide an even smaller sectioning of the viral genome.

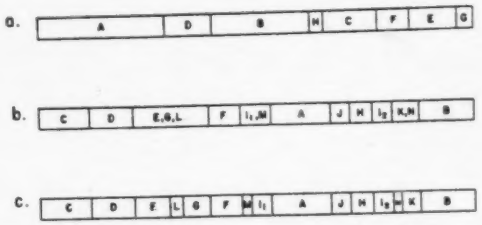

FIG. 5. *a, Physical map of SF NPV genome for Bam HI; b, partial physical map of SF NPV genome for HindIII; c, physical map of SF NPV genome for HindIII.*

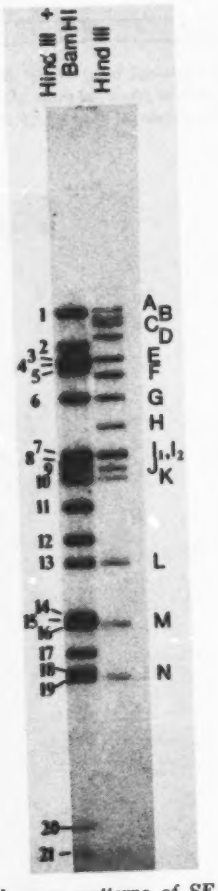

FIG. 7. *Cleavage patterns of SF NPV DNA by HindIII and (HindIII + BamHI). The restriction fragments were labeled with [α-^{32}P]dATP in the presence of DNA polymerase I.*

TABLE 4. *Molecular weights of HindIII-BamHI double-digest fragments*

Fragment	Mol wt (10^6)
1	11.1
2	7.8
3	7.0
4	6.7
5	6.4
6	5.3
7	3.9
8	3.8
9	3.7
10 (2 comigrating fragments)	3.5
11	3.1
12	2.7
13	2.4
14	1.9
15	1.8
16	1.8
17	1.6
18	1.5
19	1.4
20	0.72
21	0.59

TABLE 5. *Sequence homology between the HindIII-BamHI double-digestion products and HindIII or BamHI restriction fragments*

Double-digest fragment	*Hind*III fragments with homologous sequences[a]	*Bam*HI fragments with homologous sequences[b]
1	A	B
2	C	E
3	E	A
4	D	A
5	B	F
6	G	A
7	H	B
8	I_1	D
9	J	B
10 (2 comigrating fragments)	F, K	D, C
11	B	C
12	F	A
13	L	A
14	I_2	H
15	I_2	C
16	L[c], M	A[c], D
17	C	G
18	D	G
19	N	C
20	B[d]	E
21	H[d]	H

[a] From cross-blot hybridization data.

[b] From restriction map alignment.

[c] The spot at the point of intersection of fragment 16 and fragment L suggests a possible sequence homology between *Hind*III fragments L and M (i.e., fragment 16 in the double digest). This is already indicated in Table 2.

[d] These are deduced by comparing the molecular weights of complete *Hind*III fragments and their double-digestion products as well as restriction map positions.

TABLE 6. *Molecular weights of the BamHI, HindIII, and EcoRI restriction fragments*

Fragment	Mol wt (10^6)
*Bam*HI	
A	24.1
B	18.5
C	10.2
D	9.1
E	8.8
F	6.0
G	3.2
H	2.6
*Hind*III	
A	11.3
B	10.2
C	9.6
D	8.4
E	6.9
F	6.1
G	5.3
H	4.4
I_1	3.9
I_2	3.9
J	3.6
K	3.5
L	2.4
M	1.8
N	1.4
*Eco*RI	
A	8.1
B	7.1
C_1	6.7
C_2	6.7
D_1	6.1
D_2	6.1
D_3	6.1
E	4.8
F	3.8
G	3.3
H	2.4
I	2.4
J_1	2.0
J_2	2.0
K	1.5
L	1.4
M	1.3
N_1	1.2
N_2	1.2
O	1.2
P	1.1
Q	1.0
R	0.90
S	0.87
T	0.82

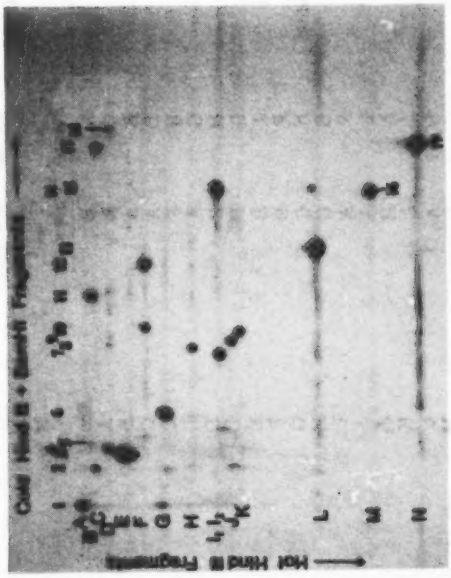

FIG. 8. *Autoradiograph of the patterns resulting from the cross-blot hybridization between ^{32}P-labeled HindIII fragments and cold HindIII and BamHI double-digested fragments of SF NPV DNA.*

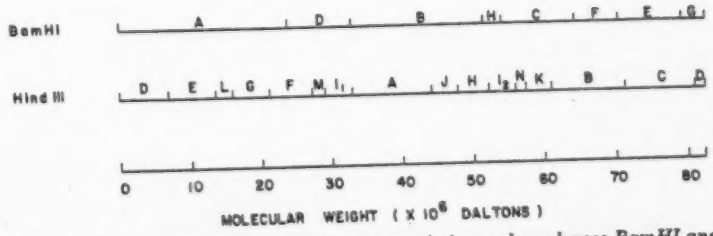

FIG. 9. *Physical maps of the SF NPV genome for the restriction endonucleases BamHI and HindIII. The BamHI fragment A is arbitrarily placed at the origin of the linear map for the sake of simplicity. The actual map should be circular.*

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Section D: Construction of a Cloned Library of the HindIII DNA Fragments of *Spodoptera Frugiperda* Nuclear Polyhedrosis Virus Genome and Mapping of Novel Fragments

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ABSTRACT

Cleavage of *Spodoptera frugiperda* nuclear polyhedrosis virus (SfNPV) Ohio Strain, DNA with restriction endonuclease HindIII yields 18 fragments ranging in size from 0.65 x 10^6, to 11.3 x 10^6 daltons. Among these 18 fragments two with molecular weights of 0.65 x 10^6, 0.93 x 10^6 and 0.68 x 10^6, respectively were not found previously. A cloned library of HindIII fragments of this strain was constructed using the plasmid pBR322 and the recipient bacterium *Escherichia coli* strain LE392. Blot hybridization was used to determine the viral origin of the cloned inserts. The library is representative of 100% viral genome. The nick-translated recombinant plasmids were used to localize the map region of the three newly discovered HindIII fragments. The physical map of HindIII SfNPV DNA fragment was confirmed.

INTRODUCTION

More than 400 baculovirus species were isolated from many host insects. Only a few of these viruses have been used as biological pesticides in agriculture and forestry. In the process of selecting and developing safe and effective pesticidal viruses, information on the molecular biology and genomic structure of baculoviruses have been obtained. Baculovirus genomes have been analyzed with restriction endonucleases (11,12), and the restriction maps of *autographa californica* NPV-DNA have been reported (4). It is expected that genetic manipulation of baculoviruses will be reported in the near future in attempts to develop improved viral pesticides.

SfNPV, a member of the baculovirus group, is one of the model systems for the study of the molecular biology of insect viruses. It has a double stranded, circular supercoiled DNA genome with a molecular weight of 80 x 10^6 (14,2,3,7). The characterization of the SfnPV genome and the physical map for BamHI and HindIII fragments of SfNPV genome have been published previously (8,9). In this communication we report a cloned library of SfNPV HindIII fragments generated in the pBR322 vector system. These recombinant plasmids are available for propagation of SfNPV DNA restriction fragments in large quantity for molecular biological experiments. During the cloning of of SfNPV HindIII DNA fragments, three additional small DNA fragments were uncovered. A revised restriction fragment map with the three additional DNA fragments is presented.

MATERIALS AND METHODS

Purification of viral DNA and plasmid DNA. The procedure for purification of SfDNA has been previously described by Loh et al. (8,9).

The plasmid pBR322 was extracted from bacteria by alkaline lysis as described by T. Maniatis et al. (10) with some modifications. Briefly, the bacterium E.coli LE392 containing plasmid pBR322 was grown in 1 liter of M9 medium (Na_2HPO_4 6g, KH_2PO_4 3g, NaCl 0.5g, NH_4Cl 1g and supplement with final concentration of 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2% glucose, 0.12% casamino acid, 1 µg/ml of thiamine, 100 µg/ml of ampicillin) at 37°C on a shaker. Until the culture reached early log phase ($O.D._{600}$ = 0.6) 150 µg/ml of chloramphenicol was added. The bacteria was incubated overnight. The cell pellet was suspended in 10 ml of ice-cold solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8) and 4 mg/ml lysozyme. After 10 min, 20 ml 0.2N NaOH and 1% SDS was added and mixed gently. For an additional 10 min on ice 16 ml of 3M Na-acetate solution was added. The lysate was centrifuged on a Sorvall GSA rotor at 12,000 rpm (23,000 g). The supernatant was extracted three times with phenol/chloroform (1:1) and then ethanol precipitated. The supercoiled plasmids were further purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradient (density 1.56 gm/ml with 500 µg/ml of EtBr).

Construction and transformation with recombinant plasmids. Plasmid pBR322 contains a single HindIII site, which interrupts the tetracycline resistance gene but does not affect the ampicillin resistance gene. HindIII-cleaved pBR322 was subjected to E. coli alkaline phosphatase digestion at a concentration of 30 units per p mole of 5'-phosphate ends in 10 mM Tris-HCl, pH 8.0, at 65°C for 30 min to prevent self-ligation of the vector during the recombination reaction. HindIII restricted viral DNA fragments were ligated to the cloning vector by incubating 1 µg of SfNPV-DNA fragments, 0.1 µg of pBR322 and 2 units of T4 ligase (BRL) for 18 hr at 12°C in a ligation buffer (100 mM NaCl, 0.1 mM ATP, 0.25 mM $MgCl_2$, 10 mM DTT, 50 µg/ml BSA, 50 mM Tris-HCl and pH 7.5) in a total volume of 15 ul.

Transformation of LE392 cells with recombinant plasmid was done according to Dagert and Ehrlich (11). The strain LE392 was cultured in L-broth (1% trypsin, 0.5% yeast extract, 0.5% NaCl pH 7) at 37°C on shaker for 3-4 hr until A_{650}=0.2 was reached. After chilling on ice for 20 min, the culture was pelleted at 3000 rpm (IEC Model HN-SII centrifuge) for 10 min. The bacterial pellet was suspended in CAST solution (50 mM $CaCl_2$, 10 mM NaCl, and 25 mM Tris-HCl, pH 7.5) and held in ice bath without agitation. After 20 min the cells were recentrifuged and resuspended in a small volume of ice cold CAST solution. Cells were used immediately for transformation.

To 0.1 ml of treated cells, 15 µl of ligation mixture containing recombinant plasmid was added. After 10 min on ice the samples were incubated at 42°C for 2 minutes, and at 37°C for 10 min in a water bath, and then chilled in ice bath again. Two ml of L-broth were added and samples were agitated at 37°C for 1 hr to allow plasmid gene expression. A sample of each culture was spread on appropriate selection plates containing antibiotics. The transformed colonies were screened by a hybridization procedure as described by Grunstein and Hogness (6).

Characterization of recombinant plasmids. Each recombinant colony detected by hybridization against nick translated ^{32}P SfNPV-DNA was amplified in LB broth containing 100 µg/ml of ampicillin for further restriction enzyme analysis. The rapid mini-prep screening procedure described by Birnboirn and Doly was used to identify isolated recombinant plasmids. HindIII digested recombinant plasmid and restriction fragments of SfNPV-DNA were separated by electrophoresis in 1% agarose gel in E buffer (0.04M Tris-HCl pH 7.2, 0.02M Na-acetate and 1 mM EDTA). For critical identification of viral DNA inserts, the restricted DNA fragments of recombinant plasmids were end-labeled in vitro with Kornberg's DNA polymerase. Each sample containing 5-30 ng DNA was incubated in 100 mM Tris-HCl pH 7.5, 20 mM NaCl, 5 mM $MgCl_2$, 6 mM β-mercaptoethanol, 0.1 mM dCTP, dGTP, TTP, 2 µCi [$\alpha^{32}P$] dATP and 0.5 unit polymerase at room temperature for 5 min and then subjected to 1% agarose gel electrophoresis in E buffer. After electrophoresis the gel was dehydrated by vacuum on a gel dryer and exposed to Kodak X-Omat R x-ray film. DNA inserts of the recombinant plasmid were identified according to migrating distance of the DNA fragments

The recombinant clones were also identified by Southern blot hybridization. After electrophoresis the restriction fragments of SfNPV-DNA were transferred onto nitrocellulose paper by the Southern technique (13). Hybridization of nick-translated SfNPV DNA to Southern filter was accomplished by incubating the filters at 65°C for 18 hr in 6xSSC, 50 µg/ml of yeast RNA, 50 µg/ml of calf thymus DNA, 25 µg/ml of polyA, 2.5 x Denhardt's solution and 0.5% SDS. After hybridization, filters were washed in 2xSSC for 1 hr at room temperature, and then 3 times washing in 0.1xSSC-0.1% SDS for 20 min at 55°C. The filter was air dried and then exposed to x-ray film.

RESULTS

Restriction enzyme HindIII digest of viral DNA. As shown in Fig. 1 and Table 1 the HindIII digestion of SfNPV genome produces 18 fragments ranging in size from 0.65 to 11.3 x 10^6, as estimated by comparing their mobilities in 1% agarose gel with standard phage λDNA fragments digested by HindIII. HindIII DNA fragments O, P and Q which were not identified in the previous report (8) have been resolved in the present study by end labelling with [α-^{32}P]dATP. Therefore HindIII-digestion yields 18 instead of 15 DNA fragments. Results of BamHI-digestion of SfNPV DNA in this study are in agreement with those of the previous report (8) as is the BamHI fragment map. To map the newly identified HindIII O, P and Q fragments, the DNA of individual clones of recombinants from HindIII-digests of SfNPV DNA and pBR322 plasmid DNAs were nick-translated with [α-^{32}P]dATP and hybridized to BamHI-cleaved SfNPV DNA. The results confirmed that the alignment of 15 fragments (A, B.... and N) deduced in the previous report was correct (8). Table 2 lists the hybridization of ^{32}P-labelled HindIII DNA fragments to cold BamHI fragments and the possible linkage of HindIII fragments deduced from the cross hybridization shown. The linkages of HindIII DNA fragments deduced are ''D-(E, L, G,)-F-(M, O) I_1 and A-J-H-I_2-(N, K)-B-(P, Q)-C-D''. Our previous mapping results demonstrate the relative fragments order (E, L, G), (M, O) and (N, K), which indicates a DNA fragment order of ''D-E-L-G-F-M-O-I_1 - A-J-H-I_2-W-K-B-(P, Q)-C-D''.

hybridization shown. The linkages of HindIII DNA fragments deduced are ''D-(E,L,G,)-F-(M,O) I_1 and A-J-H-I_2-(N,K)-B-(P,Q)-C-D''. Our previous mapping results demonstrate the relative fragments order (E,L,G), (M,O) and (N,K), which indicates a DNA fragment order of ''D-E-L-G-F-M-O-I_1 - A-J-H-I_2-W-K-B-(P,Q)-C-D''.

Fig. 1. HindIII digestion fragment pattern of purified SfnPV DNA. Restricted fragments were end-labeled with [α-^{32}P]dATP and separated by electrophoresis on a 1% agarose gel. Three additional DNA fragments O, P and Q were resolved by this method.

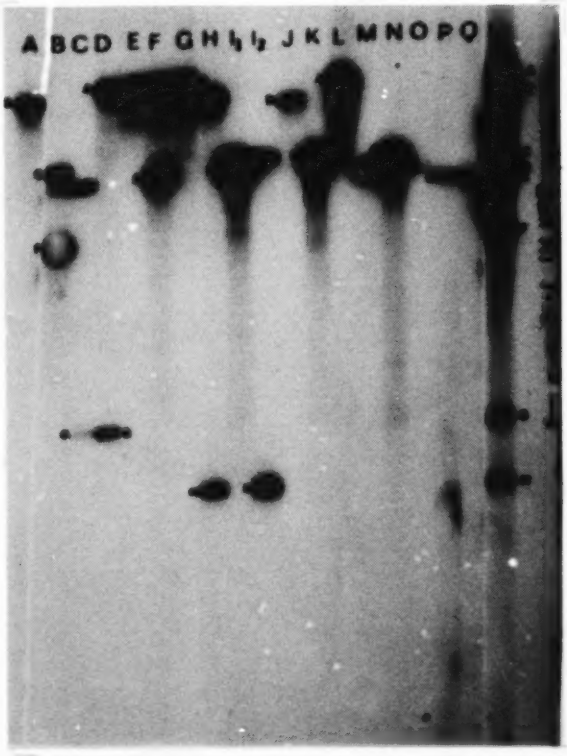

Fig. 2. Hybridization of cold BamHI-digested SfNPV DNA with α-^{32}P dATP labeled recombinant plasmid with HindIII F, I, M and O DNA inserts.

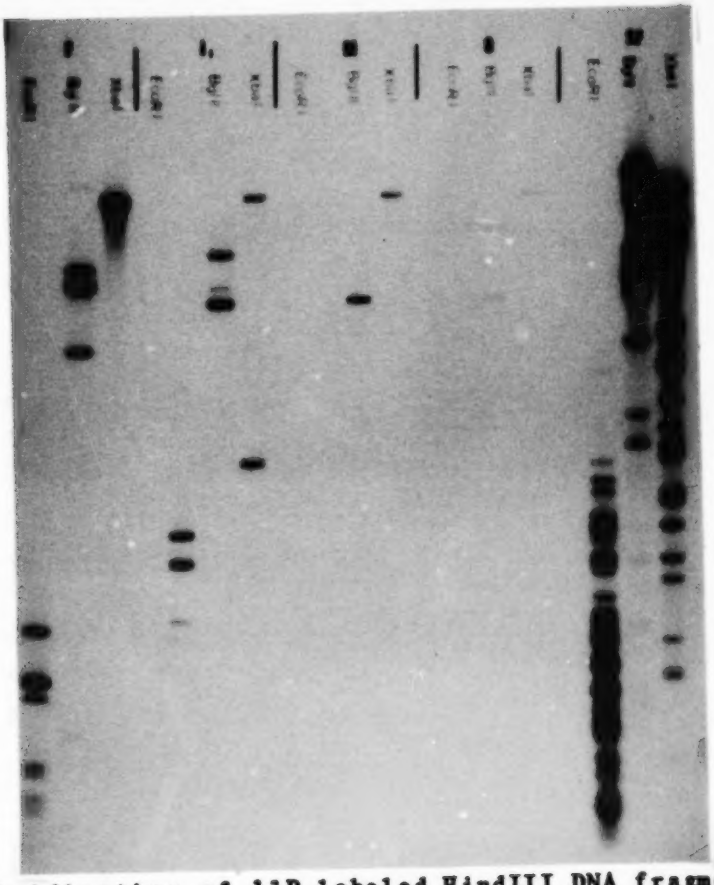

Fig. 3. Hybridization of ^{32}P labeled HindIII DNA fragments F, I_1, M and O to restricted SfNPV DNA (EcoRI, BglII, and XbaI cleaved total DNA subset). EcoRI, BglII, or XbaI cleaved SfNPV DNAs were hybridized with [-α-^{32}P]dATP-nick-translated SfNPV viral DNA or cloned F, I, M, and O fragments. As shown below, HindIII fragments F,O shared sequence homologies with BglII-digested SfNPV DNA.

The location of the M and O fragments were also simultaneously determined by hybridization of ^{32}P labelled M and O DNA fragments to whole sets of SfNPV DNA fragments generated by EcoRI, BglII and XbaI digestion (Fig. 3). Due to the small size and close linkage of the O and P fragments, their exact orientation has not been defined. Based on the intensity of hybridization to BamHI E fragment, it is suggested that HindIII Q is located next to Hind C fragment. The updated physical maps of the SfNPV BamHI and HindIII DNA fragments is summarized in Fig. 4. The actual map should be circular.

Construction of the cloned library. The first cloning was attempted with unfractionated, total restriction fragments of SfNPV DNA. The fragments were ligated to the plasmid pBR322 and used to transform to E. coli LE392. The bacterial colonies were screened on appropriate plates containing either ampicillin or ampicillin plus tetracycline. Transfection with 0.1 µg of SfNPV DNA yielded 3000 colonies of ampicillin resistant and tetracycline sensitive

(ArTs) phenotype recombinants. By colony hybridization 78 positive blots were obtained from 986 colonies. Recombinant plasmids were analyzed by electrophoretic mobility. Thirteen recombinant plasmids with inserts representing 60% of the genome. The remainder of 5 genomes were subcloned. HindIII cleaved viral DNA was subjected to electrophoresis on 1% agarose gel. The desired fragments were cut from the gel and electrophoresed. After phenol and chloroform extraction the DNA fragments were further cleaned by passing through a RPC-5 analog mini-column (BRL) to get rid of sulfated polysaccharides which are potent inhibitors of ligase. The purified individual fragments were ligated to pBR322. In this way clones carrying the remaining HindIII DNA fragment inserts were obtained. Analysis of each of 18 recombinant plasmids by HindIII enzyme is shown in Fig. 5 a and b. Recombinant plasmids with inserts of more than one viral DNA fragment were frequently obtained but are not shown.

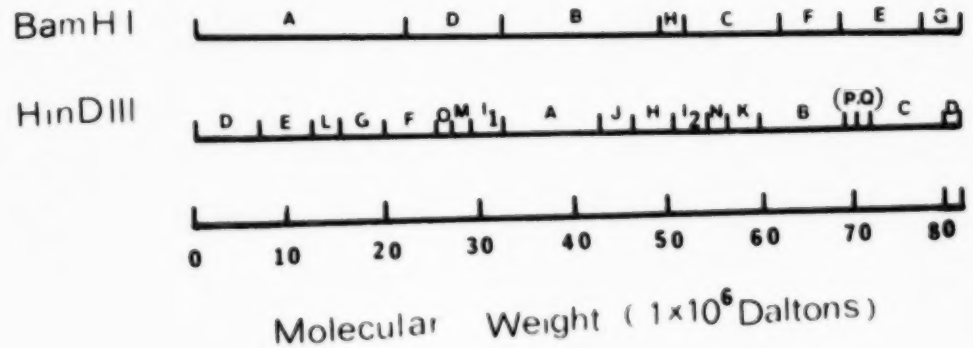

Fig. 4. Physical maps of the SfNPV genome for restriction endonucleases HindIII and BamHI. The actual map should be circular.

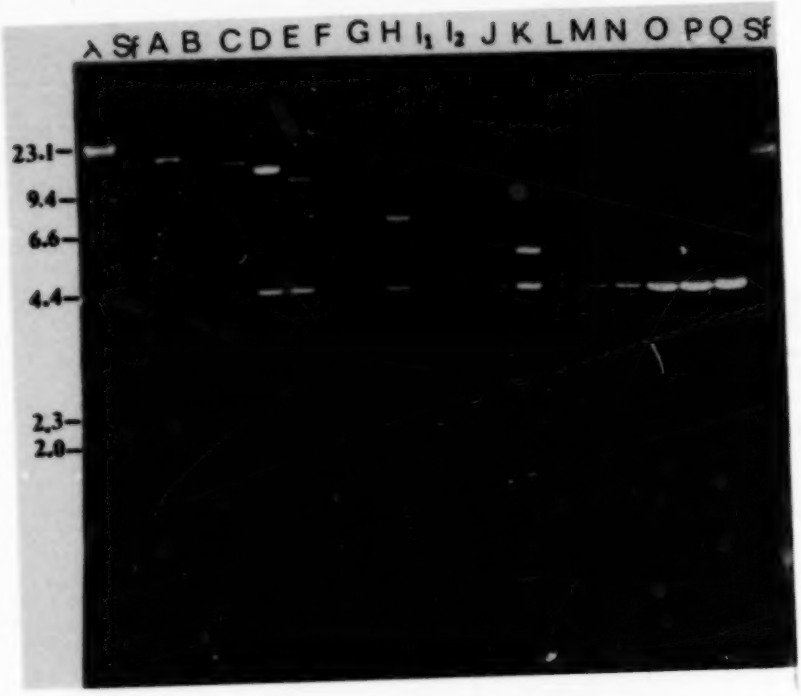

Fig. 5a. The library of cloned recombinant plasmids. The recombinant plasmid DNA was cut with HindIII and subjected to electrophoresis on 1% agarose gel. DNA was visualized by ethidium bromide stain. A,B,C....Q represents individual recombinant plasmids. The band common to all the digested plasmids is linear pBR322. =lambda DNA standard.

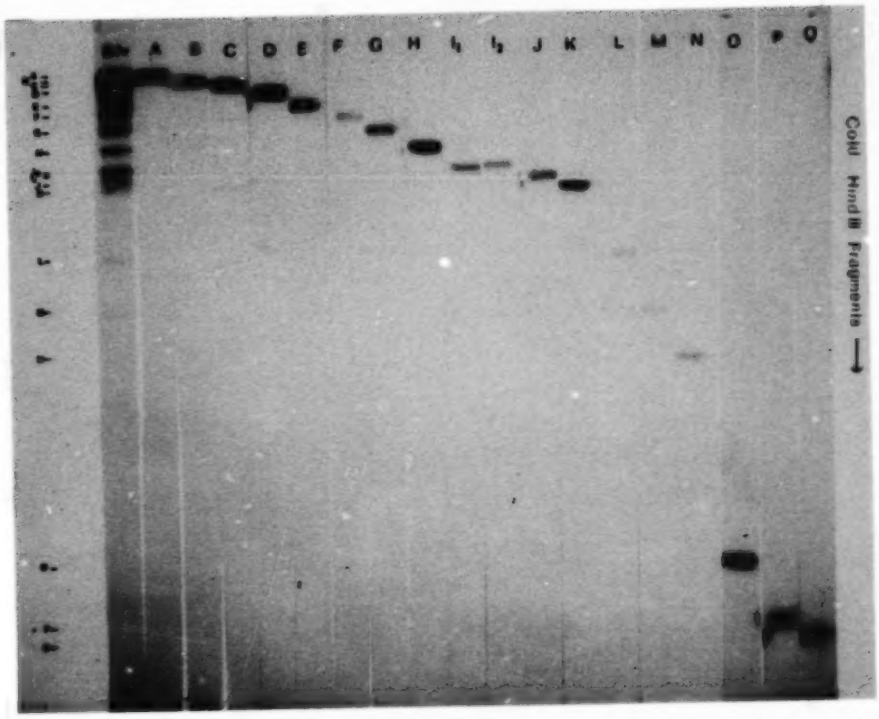

Fig. 5b. Authentication of viral DNA inserts. SfNPV DNA was cleaved with HindIII, preparatively electrophoresed in 1% agarose and blotted onto nitrocellulose filter. The filter was cut into strips. Strips were separately subjected to hybridization with individual [α-^{32}P]dATP-nick-translated recombinant cloned DNA.

DISCUSSION

Due to the host specificity and the lack of *in vitro* infectivity of occluded Sf nuclear polyhedrosis virus, the molecular biological study of this virus has been somewhat hindered. To facilitate such research, we have cloned the HindIII restricted SfNPV DNA in pBR322. The size of DNA inserts in these recombinants ranges from molecular weight of 0.65 to 11.3 million. The recombinant clones pSFP and pSFQ, which carried the HindIII P and Q inserts, respectively, were somewhat difficult to obtain. Several ligations and cloning experiments were done before stable recombinant clones were obtained. There was no difficulty in generating recombinant clones with inserts of more than 15 Kb such as the largest HindIII fragment of SfNPV DNA. During the selection and identification of the recombinants clones, inserts possessing small deletions were found. Such recombinant clones were problematic because, like complete inserts they were ampicillin resistant, tetracycline sensitive and hybridized to the original SfNPV DNA fragments. The mechanism of deletion in these recombinants and the most frequent deletion site(s) remains unknown.

$pSFI_1$ and $pSFI_2$ carry nearly identical sized fragments of about 3.9 Kb. Hybridization of ^{32}P-labelled DNA of $pSFI_1$ and $pHD20I_2$ to SfNPV BamHI DNA indicates that they are two distinct DNA fragments (Fig. 2). Separation of these two fragments could be achieved only through cloning.

HindIII DNA fragments O, P and Q were overlooked previously because of over electrophoresis and loss of the small DNA fragments. By end labelling of the SfNPV DNA fragments and shortening the electrophoresis, the three additional DNA fragments were discovered. The location of the P and Q fragments were mapped to between the HindIII B and C fragments. Presently we do not have conclusive data to determine if the actual order of P and Q is -B-P-Q-C or -B-Q-P-C-. Based on the hybridization of ^{32}P HindIII DNA fragments B, C, P and Q to BamHI E fragment (as shown on Fig. 2) is believed to be located next to Hind C fragment and that the former order (-B-P-Q-C) is the correct one. The DNA fragment map shown in Fig. 4 is linear but the data indicates that the actual map is circular.

TABLE 1

Molecular Weights of SfNPV DNA Fragments Cleaved by HindIII and Recombinant Clones Carried Corresponding DNA Fragments

HINDIII SfNPV DNA FRAGMENTS	MOL. WT. (10^6) OF DNA FRAGMENTS	RECOMBINANT CLONES WITH SfNPV DNA INSERTS
A	11.3	pSFA
B	10.2	pSFB
C	9.6	pSFC
D	8.4	pSFD
E	6.9	pSFE
F	6.1	pSFF
G	5.3	pSFG
H	4.4	pSFH
I_1	3.9	$pSFI_1$
I_2	3.9	$pSFI_2$
J	5.6	pSFJ
K	3.5	pSFK
L	2.4	pSFL
M	1.8	pSFM
N	1.4	pSFN
O	0.93	pSFO
P	0.68	pSFP
Q	0.65	pSFQ
TOTAL	86.96	

TABLE 2

Southern Blot Hybridization of ^{32}P labelled HindIII Fragments to Unlabelled BamHI Fragments and the Linkage Map of DNA Fragments

HindIII DNA Fragment ($P^{32}P$ Labelled)	BamHI Fragments with Homologous Sequences	Linkage Groups Deduced for HindIII Fragments
A	B	A-J-H
B	C, F, E (less intense)	I_2-(N, K)-B
C	E, G	B-(P, Q)-C
D	A, G	C-D
E	A	D-(E, L, G)-F
F	A, D	D-(E, L, G)-F-(M, O, I)
G	A	D-(E, L, G)-F
H	B, H	A-J-H
I_1	D	F-(M, O)-I^1
I_2	C, H	H-I_2-(W, K)-B
J	B	A-J-H
K	C	I_2-(N, K)-B
L	A	D-(E, G, L)-F
M	D	F-(M, O)-I_2
N	C	I_2-(N, K)-B
O	D	F-(O, M)-I_2
P	E	B-(P, Q)-C
Q	E	B-(P, Q)-C

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Section E: Interaction of *Spodoptera frugiperda* NPV With Various Mammalian Cell Lines *in vitro*

ABSTRACT

Detectable viral gene expression and viral nucleic acid synthesis were not observed in SfNPV or viral DNA treated mammalian cell lines, including diploid WI-38 human fibroblasts, HEL cell, NIH3T3 cells and guinea pig embryonic fibroblast. Positive expression of SfNPV antigens were demonstrated in virus infected homologous host SF 140AE cells. No positive transformation of human fibroblasts by SfNPV and its DNA were found.

INTRODUCTION

The interaction of *S. frugiperda* NPV (SfNPV) with various mammalian cell lines was studied by (a) viral structural antigens expression in virus infected or viral DNA trnsfected permissive insect cell line (S.F. cell line 140 AE and IPLB, 21AE) and non-permissive various mammalian cell lines, (b) viral DNA synthesis by ^{3}H cRNA-DNA membrane hybridization, (c) viral mRNA synthesis by *in situ* ^{32}P DNA-RNA hybridization, and (d) the oncogenicity of viral DNA by transformation of human fibroblasts (WI-38 and HEL), mouse cell 3T3 and hamster embryonic cells with SfNPV DNA by calcium phosphate and DMSO treatment. To date, we have not been able to demonstrate SfNPV viral gene expression in non-permissive mammalian cell lines using either virus from hemolymph of infected larvae or purified DNA. In contrast, positive results were found with permissive S.F. 140 AE cells. Rapid degradation of transfected DNA was found in SfNPV DNA transfected WI-38 cells.

MATERIALS AND METHODS

Cells. The human diploid WI-38 fibroblast and the HEL embryonic lung cell lines were obtained from ATCC and Dr. F. Rapp, respectively.

Two additional mammalian cells, NIH 3T3 mouse cell line and guinea pig embryonic fibroblast were also used for the infection and gene expression studies. The cells were grown in D-MEM supplemented with fetal calf serum (6 to 10%), penicillin and streptomycin at 100 units and 100 µg per ml, respectively.

Insect cell lines. *Spodoptera frugiperda* (Strains 140AE and IPLB, 21AE), were grown in Hank's medium with 10% fetal calf serum at 28°C.

Virus strains. Three *S. frugiperda* NPV strains (Ohio, Georgia and North Carolina) were used for this study. The Ohio strain was used predominantly for the DNA characterization. The virus used for infection came from the hemolymph of virus infected Sf larvae.

Synthesis of viral complementary RNA (cRNA) The viral complementary RNAs (cRNAs) were synthesized and labelled with ^{3}H UTP as described previously (3).

Radioisotope labeling of viral DNA in vitro with Kornberg's enzyme. For in situ ^{3}H or ^{32}P DNA hybridization to detect viral specific mRNA, the nick translation method was used. This method for labelling with ^{3}H TTP or ^{32}P XTP was described previously for human cytomegalovirus system. The specific activity of DNA obtained was about 2 x 10^{6} cpm/μg for ^{3}H and 1-2 x 10^{8} cpm/μg for ^{32}P. Before being used as a radioactive probe, each in vitro enzymatic labeled product was characterized for specific activity and sensitivity (percentage) of DNA to S_1 nuclease (native or denatured DNA). DNA preparations with low specific activity, uneven labeling and high background (if more than 5% of DNA is resistant to S_1 digestion when denatured or sensitive to S_1 digestion when it is in native state) were not used as probes for reassociation kinetics analysis.

Complementary RNA-DNA hybridization.

(i) Membrane filter. The hybridization of in vitro synthesized virus specific ^{3}H-cRNA to immobilized denatured DNA was achieved by the method of Gillespie and Spiegelman (2) as described by Huang et al. (3).

(ii) Cytohybridization in situ. This was carried out according to the procedure of Gall and Pardue (1) with modifications. Cells were hypotonically treated with either 0.1 x Hanks's solution or 0.1 x SSC for 20 min at 37°C and fixed with ice-chilled ethanol and acetic acid (3:1) for 10 minutes. The slide was then dipped in absolute alcohol three times, alkalinized in 0.07 N NaOH for 3 min, and washed by dipping in 70 and 95% alcohol three times each. 0.1 ml of viral specific ^{3}H-cRNA solution containing 5 x 10^{5} cpm was applied and covered with a cover glass and the samples kept wet with 6 x SSC. The hybridization was carried out at 66°C for 20 to 22 hours. After RNase digestion, complete washing and dehydration with alcohol, the slides were dipped in NTB emulsion (Eastman) and air-dried in a darkroom for at least 2 hours. For detection of viral specific mRNA, in vitro nick translated ^{32}P viral DNA was used (S.A. 1 x 10^{8} cpm/μg). The cells were not alkalinized; but instead were treated with proteinase K to uncover viral RNA. The hybridization was performed under the same hybridization condition as before. Excess ^{32}P DNA was removed by extensive washing with 6 x SSC and heating at 70°C for 10 min to remove unhybridized radioactive DNA. The autoradiography was carried at -20°C for periods of 1 to 2 days up to 4 weeks, depending on the specific radioactivity of the ^{3}H-labeled material after preparation as follows: The emulsion-cover slide was dipped into dioxane fluid with PPO (1% w/v) and POPOP (0.2%) for exposure. The cover slip or slide were then developed with Kodak D-19, fixed with rapid fix, and stained with giemsa for 30 min. For detection of viral messenger RNA in virus transformed cells, ^{32}P viral DNA or ^{3}H cRNA was used.

(iii) Transfection with viral DNA. The successful transfection and transformation of human embryonic fibroblast with human CMV DNA fragments in our laboratory encouraged us to study the possibility of oncogenicity of pesticidal virus DNA and the additional possibility of pesticidal virus gene expression in mammalian cells. The optimal conditions (.eg., concentration of viral and carrier DNA used, concentration of DMSO, etc.) were evaluated and established during the developmental stages of study in the permissive or homologous cell systems, and then extended to mammalian and human cells.

The following is the outline for the DNA transfection procedure which is essentially that of Stow and Wilkie (6). Intact or NPV DNA fragments were mixed with carrier salmon sperm DNA to a final concentration of 20 µg/ml in Hepes buffered saline containing 0.7 mM Na_2HPO_4. The $CaCl_2$ solution was added to the DNA solution to a final concentration of 1.25 mM. The mixture was overlayed on the pre-confluent cell culture (80% confluence). Thirty minutes after overlaying the DNA solution, an additional 10 ml of MEM (for WI-38, HEL and 3T3) or Grace's (for SF cell lines) media containing 4% fetal calf serum was added. Four hours later, the transfected cell cultures were subjected to DMSO shock (15% for 5 minutes). The cultures were then maintained in MEM or Grace's media with 2 to 4% fetal calf serum for a period of time. Viral gene expression was detected by the methods listed in the previous sections. Kinetics of viral DNA synthesis was examined with specific viral ^3H cRNA-DNA hybridization on nitrocellulose membrane as described previously (Huang and Pagano, 1977). Localization of infected or susceptible cells was approached by ^3H cRNA-DNA *in situ* cytohybridization and immunofluorescence test.

RESULTS

Resistance of mammalian cells to SfNPV infection. Anti-SfNPV serum obtained from an immunized guinea pig was used for the detection of virus specific antigen expression in SfNPV infected cell cultures by the indirect immunofluorescence test (IFA). Negative IFA was found in all four mammalian cell lines infected with virus from the hemolymph of infected insects. In contrast, viral antigen expression was readily detectable in the insect cell lines, IPLB 21AE, and SF 140AE cells, infected with virus from hemolymph, (see Figure 5-1-a). Under the phase contrast microscope, viral polyhedra were seen within insect cells where virus specific immunofluorescence was also found. (Figure 5-1-b).

Strain SF140AE insect cell lines and WI-38 human fibroblasts were used for the DNA transfection study. Monolayer cultures were infected with supercoil SfNPV DNA by the calcium phosphate precipitation DMSO method. At 4-7 days after transfection, the cell cultures were examined for viral antigen by IFA and viral gene transcription by *in situ* DNA-mRNA cytohybridization. Positive infected cells (about 1 in 10^4) were found in DNA transfected S.F. 140AE cells as detected by *in situ* cytohybridization using ^{32}P ATP labeled SfDNA as probe (for detecting RNA). However, we were not able to detect the positive transfection in WI-38 human fibroblast; either by FA or *in situ* cytohybridization. Because the transfection efficiency in the permissive cell was very low, it is certainly inappropriate to conclude that there is no viral gene expression in human fibroblasts.

The standard focus forming transformation assay was also carried out with SfNPV-DNA transfected WI-38, mouse Balb 3T3 and hamster embryonic cells. No morphologically transformed cell lines were established. The application of phorbol ester (TPA) at 20 ng/ml to DNA transfected culture did not enhance or cause morphological transformation of human fibroblasts.

Three- to four- day-old mouse-embryos were transfected with SfNPV-DNA using calcium chloride method, and reimplanted in fostered mothers for further embryonic development. From three experiments with more than 48 transfected embryos, only one normal baby was obtained. Unfortunately, in the control group, where identical procedures were used (calcium phosphate-DMSO) less than 10% (2 out of 30) embryos developed into full term babies. Therefore the failures in successful implantation and full-term gestation may be due to the calcium chloride and DMSO treatment.

Fate of transfecting SfNPV DNA. The fate of transfected SfNPV DNA in WI-38, guinea pig embryonic fibroblast and Sf 140 AE cells were further examined and quantitated by membrane cRNA-DNA hybridization. Cells in 75 cm^2 flask were transfected with 2 µg per flask of SfNPV DNA by the calcium chloride-DMSO procedure (6). Cells were lysed with 1% sodium lauryl sulfate (SDS) in 0.05M Tris-HCl, pH 9 and 0.01M EDTA at various times after transfection. DNA was extracted and immobilized on nitro-nitrocellulose for hybridization. Figures 4-2 show the relative quantity of viral DNA from various transfected cells as reflected by counts of ^3H cRNA hybridized. In SfNPV DNA transfected WI-38 and GP embryonic cells the input DNA was degraded early and remained at the background level 3-4 days after infection. In contrast, viral DNA synthesis was detectable at 72 hours after transfection in insect SF140AE cells and gradually reached a plateau at a relatively low level at 7 days.

DNA transfection offers the advantage of bypassing the membrane receptor barrier. However, the transfection efficiency of DNA is rather low compared to normal infection by intact virions. The low level of DNA synthesis in SfNPV DNA transfected SF140 AE cells might be caused by DNA degradation as well as inability to generate reinfection by progeny virus. The fate of transfected SfNPV DNA was also studied by alkaline sucrose gradient sedimentation. Extensive degradation into small DNA fragment was found particularly in SfNPV DNA transfected WI-38 and guinea pig embryonic fibroblasts.

CONCLUSION

Numerous *in vitro* and *in vivo* experiments have been done to show the species specificity and safety of the pesticide viruses. Although reports suggest the low infectivity to vertebrate cells (McIntosh and Kimura, 1974), we were not able to demonstrate replication and expression in SfNPV virion-infected and DNA transfected mammalian cells by DNA hybridization and immunofluorescence tests. Intact SfNPV DNA was used for morphological transformation of human fibroblast, in the presence and absence of tumor promoters, but no evidence of transformation was obtained. In view of the structure of nuclear polyhedrosis virus where virions are imbedded in a crystalline protein matrix, the occurrence of permissive infections of mammalian cells should be extremely rare. The nonpermissiveness of SfNPV infection of mammalian cells as demonstrated in this study further support the safety of SfNPV for use in controlling its pest host in agriculture.

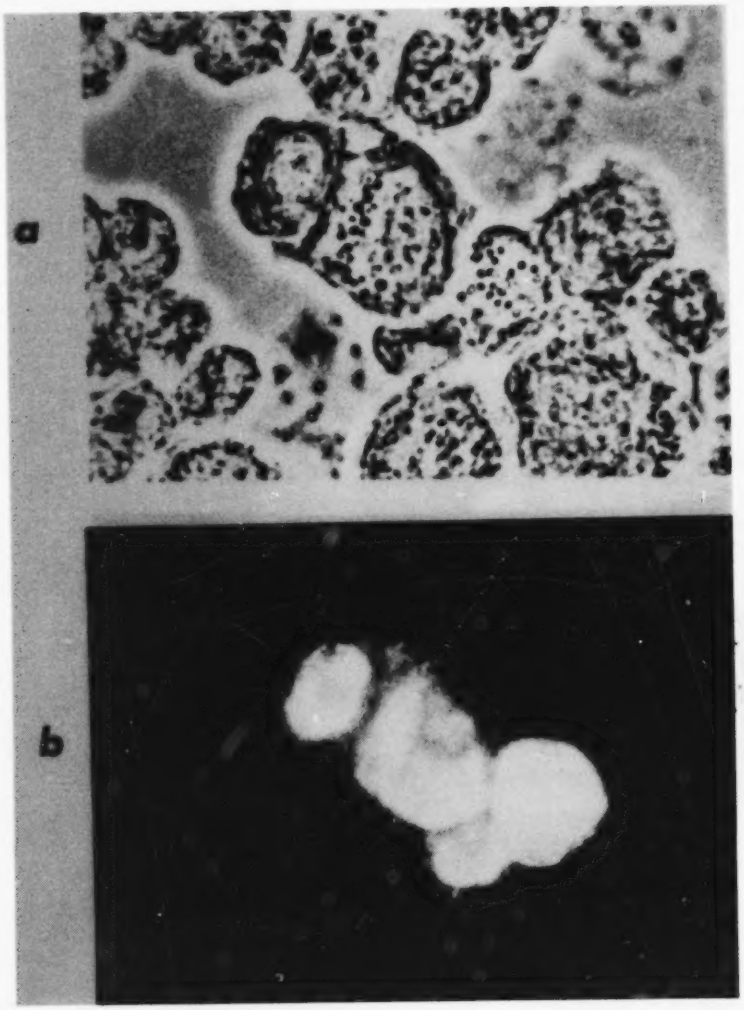

Fig. 2. Infection of Sf 140 AE cells with SfNPV from the hemolymph of SfNPV infected larvae. (a) phase contrast microscopic observation. (b) positive immunofluorescent focus.

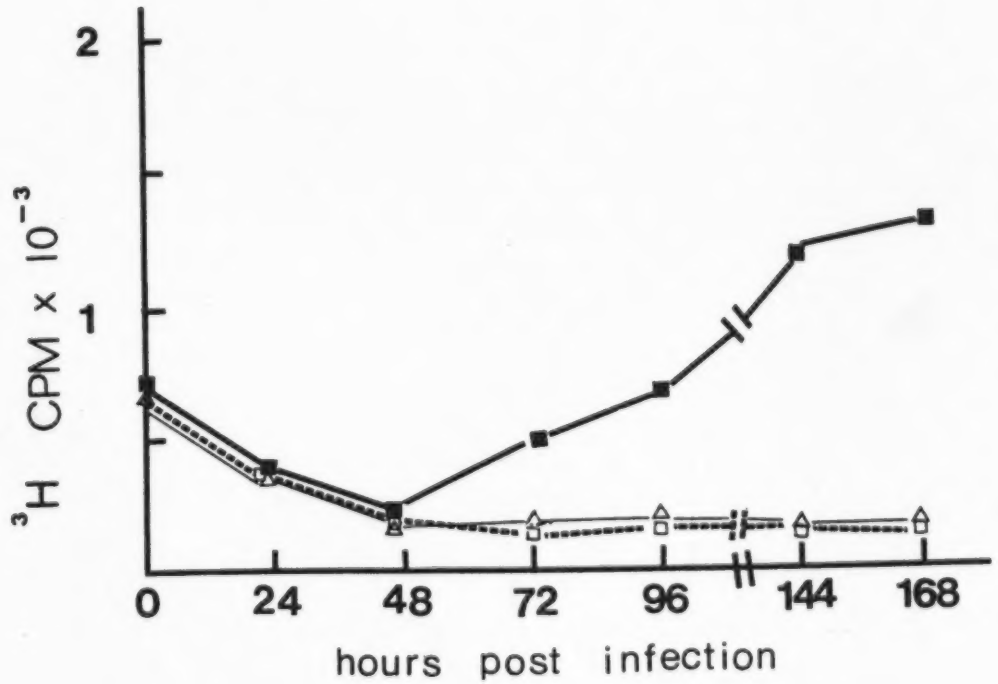

Fig. 3. Transfection of Sf 140AE cell (■), WI-38 (Δ), and guinea pig embryonic fibroblast (□) with SfNPV DNA. The DNA synthesis was detected by membrane hybridization using ^{3}H labeled cRNA of SfNPV DNA.

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